Insulin Resistance in Uremia

CHARACTERIZATION OF INSULIN ACTION, BINDING, AND PROCESSING IN ISOLATED HEPATOCYTES FROM CHRONIC UREMIC RATS

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ABSTRACT We have developed a model in the rat that leads to a predictable degree of severe uremia to study the role of the liver in the insulin-resistant state of uremia. The uremic animals were euglycemic and had increased serum immunoreactive insulin when compared with their pair-fed controls. Insulin action, binding, internalization, and degradation were characterized in freshly isolated hepatocytes from uremic animals, sham-operated pair-fed, and ad lib.-fed controls.

The basal rate of aminoisobutyric acid (AIB) uptake was increased in hepatocytes from both uremic and pair-fed control rats. However, while hepatocytes from uremic animals were refractory to insulin with regard to AIB uptake, there was no significant difference in the absolute increment above basal AIB uptake by hepatocytes from pair-fed and fed ad lib. animals at any insulin concentration studied.

¹²⁵I-Insulin binding at 24°C was higher in hepatocytes from uremic rats at every insulin concentration studied when compared with fed ad lib. controls. The time course of ¹²⁵I-insulin binding to the cell and to the fractions that were membrane bound or internalized were studied at 37°C. An increase in membranebound ¹²⁵I-insulin at 37°C was present also in hepatocytes from uremic animals. The same fraction of membrane-bound ¹²⁵I-insulin was internalized in hepatocytes from all groups of animals.

Extracellular and receptor-mediated ¹²⁵I-insulin degradation at the plasma membrane and after inter-

nalization was studied at 37°C by gel chromatography. There was a delayed and decreased rate of ¹²⁵I-insulin degradation in hepatocytes from uremic rats in the three compartments.

We conclude: (a) In chronic uremia the liver is refractory to insulin with regard to AIB uptake. (b) Insulin resistance in uremic rat liver is not due to defects in insulin binding or internalization. (c) Despite the high level of circulating immunoreactive insulin, hepatocytes from uremic rats did not show the expected "down regulation" of their insulin receptors or an increased rate of insulin degradation. These studies further emphasize the primary role of postbinding events in the regulation of insulin binding and degradation. The mechanism as to how the coordinated steps of insulin metabolism in the liver are disrupted in a pathological state is presently unknown.

INTRODUCTION

Insulin resistance is widely recognized in patients with chronic renal failure (1). Peripheral insulin insensitivity in uremia is well accepted, but the role of the liver in insulin action, binding, and processing (internalization and degradation) remains controversial or unexplored (2, 3). This is an important problem to define since the liver is a major target organ for insulin action and the main organ for insulin metabolism and degradation (4).

In order to study hepatic insulin metabolism in uremia we have developed a model of chronic renal failure in the rat that leads to a predictable degree of severe uremia. In the experimental design, specific consideration was given to the nutritional status of the animals. Two sham-operated control groups, ad lib.fed and pair-fed animals were included. This allowed

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us to analyze precisely the metabolic events due to either uremia or starvation. Finally, our ability to isolate hepatocytes that respond to (5-9) and process insulin (10-11) allowed us to characterize the relationship between insulin action and processing in uremia.

METHODS

Chemicals. α -[1¹⁴C]Aminoisobutyric acid (51.6 mCi/mmol), carrier-free Na¹²⁵I, [methoxyl-³H]inulin (186.4 mCi/g) and 3-O-[¹⁴C]methyl-D-glucose (58.0 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Crude collagenase (4177 CLSII 41K22, 164 μ m/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ, Fraction V bovine albumin from Reheis Chemical Co., Kankakee, IL, and aminoisobutyric acid from Calbiochem-Behring Corp., San Diego, CA. Crystalline porcine insulin was kindly provided by Dr. Ronald Chance of Eli Lilly & Co., Indianapolis, IN. All other chemicals were reagent grade.

Experimental model of chronic uremia. Male Sprague-Dawley rats weighing ~ 200 g were anesthesized with light ether anesthesia. The rat's flank was entered and the left kidney was separated from the adrenal gland and perirenal fat. The kidney was then decapsulated and the superior and inferior third of the kidney were ligated with 4-0 silk. The poles of the kidney became ischemic within seconds, and were surgically removed. Minor renal bleeding was controlled with mild direct pressure. The remnant kidney was placed into a chamber measuring 0.9 cm³. The chamber was made from 0.4-mm thick vinyl (Sommer's Plastic Products, Clifton, NJ). The edges of the chamber were fastened with cyanoacrylate glue or with a heat sealer. After the kidney was placed into the chamber, the open flap of the chamber was completely closed with four surgical sutures, except for a 3×3 -mm aperture for the renal pedicle. The enclosed kidney was replaced in the retroperitoneal space, and the flank closed with two layers of 3-0 Vicryl suture. 7 d after this operation, when the left remnant kidney had recovered from the stress of surgery, the right kidney was totally removed through a right flank approach leaving the right adrenal gland intact. The same technique was used to enter the retroperitoneal space of the sham-operated controls. The kidneys were then manipulated, but not removed, and a 1cm² piece of vinyl with a drop of cyanoacrylate glue was placed in the left retroperitoneal space.

Experimental protocol. Male Sprague-Dawley rats with initial weights of ~ 200 g were used for all experiments. They were maintained in a constant temperature (30°C) animal room with a fixed artificial light cycle (7:00 a.m.-7:00 p.m.). All animals were placed in individual cages and were fed standard Purina Chow (Ralston-Purina Co., St. Louis, MO).

The study animals included three experimental groups: group I consisted of uremic rats fed ad lib.; group II consisted of sham-operated rats pair fed with the uremic rats; group III consisted of sham-operated rats fed ad lib. The amount of chow given daily to any individual animal in group II was equal to the amount of chow that the pair-fed uremic animal ate during the preceding 24 h.

All animals were fasted for ~ 3 h before killing 4 wk after surgery. Their liver cells were isolated and blood was obtained by aortic puncture for measurement of blood urea nitrogen, creatinine, glucose, and immunoreactive insulin.

Preparation of hepatocytes. Liver perfusion, isolation, and suspension of hepatocytes were performed as previously

described (9). Cell viability was >90%, as was measured by exclusion of trypan blue. Furthermore, the cells incorporated ¹⁴C acetate and ³H₂O into lipids linearly over 3 h, and ¹⁴C leucine into trichloroacetic acid, precipitable material linearly over 4 h at 37°C.

Insulin action studies. The ability of insulin to stimulate the uptake of α -aminoisobutyric acid (AIB),¹ a nonmetabolizable analogue of alanine, was used to assay insulin action. Freshly isolated hepatocytes were suspended (2-4 $\times 10^6$ cells/ml) in Krebs Ringer bicarbonate buffer pH 7.4, supplemented with 3% bovine serum albumin. The cells were preincubated at 37°C in the absence or presence of varying concentrations of insulin for 2 h, as previously reported (9). After preincubation, [¹⁴C]AIB (0.1 mM, 1.4 mCi/mmol) was added to the incubation mixture. Since AIB uptake was linear for at least 20 min, the reaction was terminated at 10 min to obtain initial rates of uptake (9).

Insulin binding studies. Insulin was iodinated (1 Ci/ μ mol) with chloramine T according to the method of Cuatrecasas (12) and insulin binding was assessed at 24 and 37°C, as previously described (9), in a cell suspension containing 0.5–1.5 × 10⁶ hepatocytes/ml. Insulin binding was expressed as cell-associated ¹²⁵I-insulin after subtraction of nonspecific binding, determined in the presence of excess 1 × 10⁻⁶ M unlabeled insulin. The nonspecific insulin binding was consistently <10% in the experiments at 24°C and <30% in those at 37°C of the maximal amount bound.

Insulin internalization studies. The internalization of ¹²⁵I-insulin was analyzed by a method used previously to study the internalization of other ligands in different tissues (13-15) based on the ability of acid pH to dissociate surfacebound ligands. We have recently described a method that allows separation of insulin associated with hepatocytes into two compartments (11). Insulin removed by low pH is membrane bound and that resistant to acid pH is internalized. Briefly, after incubation with ¹²⁵I-insulin as described earlier for binding experiments, the isolated hepatocytes were washed three times with phosphate-buffered saline, pH 7.4, at 4°C to remove free ¹²⁵I-insulin. Hepatocytes were then treated at 4°C for 6 min with incubation buffer that had been adjusted to pH 3.5 with HCl. Insulin dissociated into the medium by the acid buffer representing membranebound insulin was separated from internalized insulin by rapid centrifugation of hepatocytes through oil (9).

Insulin degradation studies. Degradation of ¹²⁵I-insulin was studied under the same conditions as described previously for insulin binding and internalization. The degradation of ¹²⁵I-insulin in the incubation medium in experiments performed at 24°C was determined by precipitation with 10% trichloroacetic acid (7). For the experiments done at 37°C, samples from the incubation medium, membranebound, and internalized material were dissolved in 4 M urea, 1 M acetic acid, and 0.1% Triton X-100, and stored at -20° C. Later the samples were thawed, vortexed, and centrifuged at 10,000 g for 1 min. The supernatant was then chromatographed on a 0.9×60 -cm column of Sephadex G-50 equilibrated an eluted with 4 M urea, 1 M acetic acid, and 0.1% Triton X-100 (7), and 1.5-ml fractions were collected. I¹²⁵ recovery from the column exceeded 95%.

In each experiment, appropriate control flasks were prepared that were identical to experimental flasks in all respects, except that liver cells were omitted. The amount of insulin degraded in the control flasks was then subtracted

¹ Abbreviation used in this paper: AIB, α -aminoisobutyric acid.

from insulin degraded in the experimental flasks to estimate cell-mediated insulin degradation. The leakage of degradative enzymes into the medium accounts for <1 and 6% of the total insulin degradation observed at 25 and 37°C, respectively (7).

The amount of degraded iodinated material was calculated after gel filtration or trichloroacetic acid precipitation as: percentage of degradation products in the sample times total amount of iodinated material either present in the medium or bound to the cell. Degradation velocity was calculated by dividing the amount of insulin degraded by the length of the incubation time. Insulin degradation data represent the total amount of degradation products from both native insulin plus¹²⁵I-insulin. Furthermore, insulin degradation is correlated with total insulin binding without correction for "nonspecific" insulin binding since it has been shown that all insulin binding sites, including nonspecific sites, mediate insulin degradation (10, 16).

Cell counting, sizing, and blood measurements. Freshly isolated hepatocytes were counted in a hemocytometer. The intracellular water space was estimated utilizing 3-O-[14C]methyl-D-glucose and adjusting for the trapping of extracellular water by the $[methoxyl-{}^{3}H]$ inulin space measurement (5). Serum immunoreactive insulin was measured by radioimmunoassay at the Diabetes Research Center of the University of Pennsylvania Medical School.

RESULTS

Experimental model of chronic uremia. This new experimental model of chronic uremia using a vinyl chamber to prevent hypertropy of the remnant kidney, leads to a predictable degree of uremia. A container with a volume of 0.9 cm^3 resulted in a uremic state that was severe and uniform in 4 wk. The uremic rats were weak, lethargic, had coarse, yellowish hair that tended to fall out and some had gross and fine tremors of their limbs that were consistent with severe uremia. As shown in Table I, the uremic rats did not gain weight over the 4-wk experimental period because food intake was decreased. The final kidney mass in

 TABLE I

 Morphometrics and Serum Measurements from the Uremic, Sham-operated

 Pair-fed, and Fed Ad Lib. Control Rats

	Uremic rats	Sham-operated, pair-fed	Sham-operated, fed ad lib.
Rat body wt at 1st operation, g	206 ± 4	195 ± 8	195 ± 7
	(<i>n</i> = 60)	(<i>n</i> = 26)	(<i>n</i> = 26)
Body wt at 2nd operation, g	224 ± 4	225 ± 8	220 ± 11
	(<i>n</i> = 60)	(<i>n</i> = 26)	(<i>n</i> = 26)
Final body wt, g	224 ± 8	220 ± 9	359 ± 11
	(<i>n</i> = 28)	(<i>n</i> = 23)	(<i>n</i> = 26)
Chow intake per rat 1st to 2nd operation, g	124 ± 4	146 ± 8	149 ± 8
	(<i>n</i> = 60)	(<i>n</i> = 26)	(<i>n</i> = 26)
Chow intake per rat 2nd operation to sacrifice, g	463 ± 16	466 ± 17	811 ± 29
	(<i>n</i> = 60)	(<i>n</i> = 23)	(<i>n</i> = 26)
Weight of left kidney removed at partial nephrectomy 1st operation, g	0.34 ± 0.01 (<i>n</i> = 60)	_	_
Weight of right kidney removed at total nephrectomy 2nd operation, g	1.17 ± 0.05 (<i>n</i> = 26)	_	_
Final kidney mass at sacrifice, g	1.10 ± 0.05	2.00 ± 0.03	2.94 ± 0.06
	(<i>n</i> = 18)	(n = 23)	(n = 26)
BUN, mg/dl	105 ± 11 (<i>n</i> = 13)	23 ± 2 (<i>n</i> = 12)	19 ± 1 $(n = 14)$
Creatinine, mg/dl	2.1 ± 0.1	0.5 ± 0.05	0.4 ± 0.04
	(<i>n</i> = 9)	(<i>n</i> = 4)	(n = 5)
Glucose, mg/dl	173 ± 10	168 ± 8	193 ± 8
	(<i>n</i> = 10)	(<i>n</i> = 10)	(<i>n</i> = 10)
Insulin, $\mu U/ml$	33 ± 4 $(n = 10)$	10 ± 1 $(n = 10)$	24 ± 2 $(n = 10)$

Values are means±SEM. The values in parenthesis indicate the number of rats studied.

the uremic animals was approximately one-half of that of the sham-operated pair fed animals and one-third of that of the ad lib.-fed controls. Table I also shows that serum creatinine and blood urea nitrogen were approximately four times greater in the uremic rats than in the ad lib.- and pair-fed controls. In addition, all groups were euglycemic but the uremic animals had increased serum immunoreactive insulin especially when compared with the sham-operated, pairfed animals.

Hepatocytes from the uremic animals (water space = $3.4\pm0.9 \ \mu l/10^6$ cells) and pair-fed controls ($3.8\pm2 \ \mu l/10^6$ cells) were smaller than those from the fed ad lib. controls ($6.3\pm0.4 \ \mu l/10^6$ cells). This was partially due to differences in glycogen concentration (uremics 25 ± 4 ; pair-fed controls 17 ± 2 ; fed ad lib. controls 75 ± 6 mg of glycogen/g of liver).

Insulin action. The ability of insulin to stimulate AIB uptake in hepatocytes was used as a bioassay of insulin action (5, 7, 9, 17). The basal rate of AIB uptake was significantly increased in the uremic rats (100 ± 10) $pmol/10^6$ cells per min, P < 0.05) and the pair-fed control animals (115±26 pmol/10⁶ cells per min, P < 0.01) when compared with the fed ad lib. controls $(46\pm 6 \text{ pmol}/10^6 \text{ cells per min})$. The dose response curves for insulin-stimulated AIB uptake expressed as a percentage and absolute increase above basal are illustrated in the upper and lower panel of Fig. 1, respectively. Hepatocytes from ad lib.-fed and pairfed animals responded to insulin at concentrations from 1×10^{-9} M to 1×10^{-6} M (P < 0.02-0.001) and 1×10^{-10} M to 1×10^{-6} M (P < 0.01-0.001), respectively. In contrast, hepatocytes from uremic rats were totally unresponsive to insulin. The percentage AIB uptake above basal was significantly greater in the ad lib.-fed controls when compared with that from the pair-fed controls, at insulin concentrations from 1×10^{-8} M to 1×10^{-6} M. However, due to the enhanced basal rate of AIB uptake in the cells from pairfed animals, there was no significant difference between the two curves at any insulin concentration tested when the data were expressed as an absolute increment above basal

Insulin binding and processing. Insulin binding and processing were studied in order to ascertain their relationships with insulin action.

The upper panel of Fig. 2 demonstrates the time course of cell-associated (membrane bound and internalized) ¹²⁵I-insulin at 37°C in freshly isolated hepatocytes from uremic, pair-fed, and ad lib.-fed rats. The amount of ¹²⁵I-insulin associated with the hepatocytes increased with time and reached an apparent steady state in 10 min. 50% of maximal binding was achieved between 2.5 and 5 min in the three groups of hepatocytes; however, the magnitude of cell-associated ¹²⁵I-



FIGURE 1 Dose-response curve of insulin-stimulated AIB uptake (0.1 mM) in isolated hepatocytes from uremic (\blacksquare), pair-fed control (O) and fed ad lib. control (\bullet) rats. Initial uptake was measured at 10 min after 120 min preincubation with different concentrations of insulin. The data are expressed as the percentage (upper panel) and as the absolute amount (picomoles per 10⁶ cells/minute, lower panel) above basal (no added insulin). Basal AIB uptake was 100±10, 115±26, and 46±6 pmol/10⁶ cells per min for the uremics, pair-fed, and fed ad lib. controls, respectively). Each point is the mean±SEM from six separate experiments.

insulin bound varied from group to group. Hepatocytes from uremic rats bound significantly more insulin than the fed ad lib. controls after 5 min (P < 0.01-0.001). Although they appeared to bind more insulin than the pair-fed controls, this difference was not statistically significant. Hepatocytes from pair-fed controls bound significantly more insulin than those from fed ad lib. controls at or after 10-min incubation (P < 0.05).

Because insulin is rapidly internalized at 37°C (11), studies were performed to ascertain whether the changes in cell-associated ¹²⁵I-insulin were due to different degrees of insulin internalization. The middle panel of Fig. 2 demonstrates that the amount of insulin internalized increased for up to 10 min and then plateaued in the hepatocytes of the three groups studied. Hepatocytes from uremic animals internalized more insulin than those from the ad lib.-fed controls after 5 min (P < 0.05-0.001) and to the same extent as those from pair-fed controls. Insulin internalization was increased in the pair-fed controls (30 min) when com-



FIGURE 2 Time course of ¹²⁵I-insulin association to freshly isolated hepatocytes at 37°C. Freshly isolated hepatocytes from uremic (\blacksquare , U), pair-fed control (O, PF), and fed ad lib. control (●, AL) rats were incubated with ¹²⁵I-insulin, 1×10^{-10} , in the presence and absence of unlabeled insulin 1×10^{-6} M. At different times the association process was stopped and the amount of specific ¹²⁵I cell-associated insulin determined. The fraction that was internalized and the fraction that was membrane bound were measured as described in Methods. The upper panel demonstrates the specific ¹²⁵Iinsulin associated with the total cell; the middle panel internalized ¹²⁵I-insulin; the lower panel membrane-bound ¹²⁵I-insulin. Each point is the mean±SEM from six separate experiments.

pared with the ad lib.-fed rats (P < 0.05). The lower panel demonstrates that membrane-bound ¹²⁵I-insulin increased rapidly, reached a maximum by 5 min, and plateaued thereafter. The same relationships among the three groups of cells were encountered for membrane-bound insulin and for internalized insulin. Fig. 3 demonstrates that in the hepatocytes of the three groups studied the percentage of membrane-bound and internalized insulin changes similarly in a reciprocal relationship with time and equilibrates after 15 min.

Investigation into whether the differences in insulin binding observed were due to changes in the number of insulin binding sites, changes in their apparent affinity for insulin or both, led us to study insulin binding at apparent steady state at 24°C over a wide range of insulin concentrations. This lower temperature was utilized in these experiments in order to decrease insulin internalization and degradation. Hepatocytes from uremic animals bind more insulin than those





FIGURE 3 Time course of ¹²⁵I-insulin association to freshly isolated hepatocytes at 37°C from uremic (\blacksquare , U), pair-fed control (O, PF), and fed ad lib. control (\bigcirc , AL). The data is a replot of ¹²⁵I-insulin internalized and membrane bound from Fig 2 expressed as the percentage of the specific ¹²⁵Iinsulin associated with the total cell.

from (P < 0.05-0.001) ad lib.-fed controls at every insulin concentration tested (Fig. 4). Insulin binding in hepatocytes from pair-fed controls was intermediate to the other two groups. The concentrations of native insulin displacing 50% of tracer ¹²⁵I-insulin (1×10^{-10} M), was $1\pm0.3 \times 10^{-9}$ M, $1.3\pm1 \times 10^{-9}$ M, and $1.2\pm0.3 \times 10^{-9}$ M for the uremic, pair-fed, and ad lib.-fed controls, respectively. This suggests that the binding af-



FIGURE 4 Scatchard analysis of insulin binding. Freshly isolated hepatocytes from uremic (**I**), pair-fed control (O), and fed ad lib. control (**O**) rats were incubated with ¹²⁵I-insulin, 1×10^{-10} , in the presence of increasing concentrations of unlabeled insulin at 25°C for 45 min. The reaction was then stopped and specifically bound, and free insulin determined as described in Methods. The insert demonstrates the concentration of specifically bound insulin per 1×10^6 cells as a function of the concentration of free insulin in the medium. The data represent the mean±SEM of six separate experiments.

finity for insulin is the same in all the three groups. A similar conclusion could be drawn when the data were analyzed by the method of Scatchard (Fig. 4). From these curves, a change in the number of receptor sites appears responsible for the increased binding of insulin in uremia, not a change in the apparent affinity. The changes in insulin binding observed in these experiments at 24°C cannot be explained by difference in the concentration of intact free ¹²⁵I-insulin present in the medium. The percentage of ¹²⁵I-insulin $(1 \times 10^{-10} \text{ M})$ degraded by cells from uremics, pairfed, and ad lib.-fed controls at 24°C was 11±1, 3±1, and 13 ± 3 , respectively. It decreased with increasing concentration of unlabeled insulin. At 1×10^{-6} M insulin concentration the percentage of insulin degraded was 4 ± 1 , 2 ± 0.5 , and 4 ± 1 , respectively. In addition, if the log of the velocity of total insulin degradation was plotted against the log of the total insulin bound (Fig. 5) a linear relationship was found. This has been previously demonstrated in freshly isolated (9, 16) and primary cultures (10) of hepatocytes and suggests that



FIGURE 5 Plot of the log of the velocity of total insulin degradation (nanograms per minute) against the log of total insulin bound (nanograms/ 1×10^6 cells). Freshly isolated hepatocytes from uremic (**I**), pair-fed control (O), and fed ad lib. control (**O**) rats were incubated with ¹²⁵I-insulin, 1×10^{-10} M, in the presence of increasing concentrations of unlabeled insulin at 25°C for 45 min as in Fig. 4. Insulin degradation was evaluated by the TCA precipitation method. The data represent the mean±SEM of six different experiments.



FIGURE 6 Gel filtration profiles of ¹²⁵I material in the medium. Freshly isolated hepatocytes from uremic (upper panel), pair-fed control (middle panel), and fed ad lib. control rats (lower panel) were incubated with ¹²⁵I-insulin, 1×10^{-10} M, at 37°C for different times as shown in Fig 2. At each time an aliquot from the incubation medium was dissolved in 4 M urea, 1 M acetic acid, and 0.1% Triton X-100, and subjected to gel filtration on Sephadex G-50 in the same buffer. This figure shows representative gel profiles at 15 min. Table II summarizes the data. In each profile, from left to right, the first peak represents material eluting in the void volume as indicated by blue dextran, the second peak coelutes with insulin, and the third peak represents final degradation products that elute with or shortly before Na¹²⁵I. Individual fraction volumes equal 1.5 cm³.

degradation velocity is first order with respect to the total amount of insulin bound. Fig. 5 also demonstrates that whereas the relationship between total insulin binding and degradation is maintained in the hepatocytes of the three groups studied, hepatocytes from ad lib.-fed controls appear to degrade more insulin for a given amount of total insulin bound compared to the uremic and pair-fed control rats. This difference became more apparent when insulin degradation was analyzed by gel chromatography in the experiments performed at 37°C. As shown in Fig. 6 and Table II, chromatography of ¹²⁵I material in the medium from the experiments at 37°C present in Fig. 2 revealed three elution peaks. Peak I eluted in the void volume, peak II eluted with purified insulin, peak III eluted with or shortly before Na¹²⁵I. Insulin degradation was markedly decreased in the hepatocytes from uremic animals compared to ad lib.-fed animals. Insulin degradation was intermediate to these two groups in the hepatocytes from pair-fed rats. It should be noted that a lag of 15, 10, and 5 min exists before a major increase of iodinated insulin degradation products (peak III) in the incubation media from hepatocytes in uremic, pair-fed, and ad lib.-fed controls, respectively. Chromatography of the media from uremic hepatocytes reveals a small amount of ¹²⁵I material between peaks II and III; whereas that from pair-fed and fed ad lib. controls demonstrates a substantial amount of intermediate molecular weight ¹²⁵I material. The acid wash chemical method used in this study that discriminates between internalized and membrane-bound ¹²⁵I material allowed us to examine by gel chromatography the nature of the radioactive material in membranebound and internalized components. Table II demonstrates gel chromatography data of ¹²⁵I membrane and ¹²⁵I internalized bound material from the experiments shown in Fig. 2. Chromatography of ¹²⁵I membrane-bound material yielded a smaller peak I than that of ¹²⁵I internalized material in the hepatocytes from the three groups of animals studied. The ratio between peaks I, II, and III from internalized material does not change significantly with time. In contrast, there is a reciprocal change with time between peak II (insulin) and peak III (insulin degradation products) from ¹²⁵I membrane-bound material. Insulin degradation products although different in quantity are found in both internalized and membrane compartments. In both compartments there is a decrease in insulin degradation in the hepatocytes from the uremic animal compared to the ad lib.-fed controls, whereas degradation in the pair-fed controls falls between the two. However, as shown in Table II, the decreased and delayed appearance of insulin degradation products into the medium of hepatocytes from uremic animals appears mainly due to a significant decrease in insulin degradation at the plasma membrane site. The degraded material found at the membrane site is not a reflection of nonspecifically bound ¹²⁵I material, since the percentage of nonspecific binding in the three groups of animals is identical.

 TABLE II

 Sephadex G-50 Elution Profiles of ¹²⁵I Material in the Incubation Medium, Cell Membrane-Bound, and Internalized Compartments from Uremic, Pair-fed and Fed Ad Lib. Control Rats

	Percentage of total counts											
			Medium			Membrane bound			Internalized			
Min	Peaks	Uremic	Pair-fed	Ad lib.	Uremic	Pair-fed	Ad lib.	Uremic	Pair-fed	Ad lib.		
5	I	1.3 ± 1.2	0.1±0.1	1.2±1.2	0.5±0.5	0.6±0.6	2.0±1.0	5.5 ± 3.5	7.0±1.2	3.8 ± 2.2		
	II	96.4±0.4	97.6±1.3	96.4±1.8	99.1±0.8	96.4±1.9	89.5±1.7	83.7 ± 2.2	71.1 ± 5.5	65.7 ± 2.1		
	Ш	2.2 ± 1.2	2.3 ± 0.9	2.3 ± 0.9	0.3 ± 0.3	2.9±1.9	8.4±0.9	10.7 ± 5.3	21.8 ± 9.7	30.4 ± 0.3		
10	I	0.2±0.1	0.0±0.0	0.3±0.17	1.9±0.1	2.0 ± 2.0	2.5 ± 1.3	2.2 ± 1.1	4.3 ± 2.2	4.1±1.2		
	II	99.0±0.2	97.6 ± 1.2	87.6 ± 6.1	97.1±1.4	91.6±0.8	89.3 ± 5.6	82.8 ± 2.8	75.4 ± 8.2	67.4±3.2		
	III	0.8 ± 0.4	2.4±1.2	12.1 ± 6.3	0.9 ± 0.9	6.3±1.3	8.1±4.2	15.0 ± 1.8	20.1 ± 6.9	28.4 ± 3.2		
15	I	0.0±0.0	1.3 ± 1.2	3.6±0.0	0.8±0.8	2.7 ± 1.2	1.3±0.6	3.2 ± 0.2	6.2 ± 0.7	3.1±1.6		
	II	97.4±0.4	91.7±1.2	83.5 ± 3.5	98.2±1.7	85.4±2.6	80.1±1.6	82.6 ± 1.4	72.1±5.0	68.5±17.0		
	III	2.5 ± 0.4	2.4 ± 1.2	12.9 ± 3.5	0.9 ± 0.9	11.8 ± 2.0	18.6 ± 2.2	14.1 ± 1.7	21.4 ± 0.5	28.3 ± 2.6		
30	I	1.4±1.4	2.7 ± 2.2	4.8±0.9	2.7 ± 0.6	1.7±0.7	2.0 ± 2.0	3.6 ± 0.6	6.8±1.3	4.6±4.6		
	II	90.7 ± 2.1	76.4 ± 2.0	55.1±0.7	85.6±1.2	86.5±3.4	59.8 ± 5.6	82.7±0.8	71.3 ± 3.1	67.2 ± 10.9		
	III	7.9±1.0	20.9 ± 2.0	40.0±8.0	11.6±1.6	11.7 ± 2.7	38.1±5.2	16.1±0.9	21.9±2.2	28.1±19.8		

Values are means±SEM of three separate experiments.

Hepatocyte suspensions from uremic, pair-fed, and fed ad lib. control animals were incubated with ¹²⁵I-insulin $(1 \times 10^{-10} \text{ M})$ for different time periods at which time the ¹²⁵I material from the incubation medium, cell membrane bound, and internalized compartments were separated as described in Methods. Peak I represents material eluting in the void volume, peak II coelutes with insulin, and peak III represents final degradation products that elute with or shortly before Na ¹²⁵I.

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DISCUSSION

The present studies were undertaken to evaluate at the cellular level the role of the liver in the insulin-resistant state of uremia. We developed a new model of chronic uremia in the rat that produced a consistent degree of severe uremia. Uremic animals were compared with two groups of sham-operated animals, ad lib.-fed and pair-fed with the uremic animals. Both control groups were used to differentiate between the metabolic derangements due to malnutrition and uremia. The relationships between insulin action, binding, and processing were then studied in freshly isolated hepatocytes from these three groups of animals. The uremic animals were euglycemic and had an increased serum immunoreactive insulin when compared with the pair-fed controls (Table I). This is suggestive of an insulin-resistant state. It should be recognized, however, that the elevated serum immunoreactive insulin present in the uremic animals could partially result from the known disproportionate increase of proinsulin in uremia that may cross react with the insulin antibodies used in the radioimmunoassay (18). Also, the animal studied had a severe degree of uremia and they could have had multiple defects not evaluated. i.e., electrolytes, state of hydration, blood pressure and altered hepatic hemodynamics, which could partially be responsible for the altered insulin metabolism observed.

The basal rate of AIB uptake was significantly increased in the uremic rats and the pair-fed control animals when compared with the fed ad lib. controls. The basal rate of AIB uptake has been reported to be increased in hepatocytes from 48- to 72-h fasted rats (5, 17) and in diabetic rats (5). This has been attributed to the appearance of a high affinity transport system for amino acids that has the properties of a pure "A" system (17). Our data are consistent with these observations and suggest that such a transport system may play a regulatory role in the control of gluconeogenesis in prolonged starvation. It is likely that the increased basal AIB uptake in uremia is secondary to starvation and not due to uremia. However, parenteral hyperalimentation studies in the uremic animals will be necessary to conclusively answer this question.

The differences in the net basal AIB uptake in the different groups complicate the interpretation of the hepatocytes' responsiveness and sensitivity to insulin. For example, in the pair-fed controls, the insulin-stimulated AIB uptake, expressed as a percentage change above basal, was significantly lower when compared with that of the ad lib.-fed controls. However, the absolute increment above basal AIB uptake in response to insulin in both groups was similar because the net basal AIB uptake was higher in the pair-fed controls compared with the ad lib.-fed group (Fig. 1). It is clear, however, that the hepatocytes from uremic animals were totally refractory to insulin, because although they started at a high basal value it should have been possible for them to have reached even higher levels (i.e., to the maximum value obtained in the pair-fed animals).

It is important to emphasize that the present study only pertains to AIB uptake and does not imply that the uremic liver is universally refractory to insulin. Future studies will determine the role of uremia in lipid and carbohydrate metabolism. This is particularly important since in a given metabolic state, one tissue but not another may be resistant to insulin (19, 20). Furthermore, a given cell may be resistant to one but not other hormone actions (21). Therefore, the designated "hormone-resistant state" should be qualified for each specific tissue and individual hormone action.

Insulin binding was studied to ascertain its relationship with insulin action. It is clear from the studies at 37 and 24°C (Fig. 4) that insulin binding is increased in uremia when results are compared with sham-operated ad lib.-fed controls, and that insulin binding in the sham-operated pair-fed controls is intermediate to the other two groups. Furthermore, if the insulin binding data are expressed per surface area the difference between the uremic and pair-fed control animals and ad lib.-fed controls is enhanced since hepatocytes' intracellular water space from ad lib.-fed animals is larger than the other two groups. This information is of particular interest since it has been suggested that the number of insulin receptors in target tissues and the serum insulin concentration are inversely related ("down regulation"). This has been demonstrated in humans and animal in different metabolic states (22-24). Studies in vitro using IM-9 lymphoblastoid cells (25), fibroblasts (26), adipocytes (27), and hepatocytes (6, 28) have clearly shown inverse relations between insulin and the concentration of its membrane receptors. Our studies and those of others (5, 29, 30) showing an increase in insulin binding in hypoinsulinemic starving subjects is consistent with the above proposed model. There are however, several examples (31-37) in which normal numbers of insulin receptors are associated with high insulin concentrations and resistance to insulin. Our uremic animals are insulin resistant and appear to be hyperinsulinemic, yet their hepatocytes have increased insulin binding. Caro and Amatruda (6) have recently proposed that down regulation of the insulin receptor may be a complex biological response to insulin. Thus, resistance of cells to this effect may explain how a target cell from a patient or animal can have normal or high number of insulin receptors in the presence of increased plasma insulin concentrations. Down regulation of the insulin receptor has been proposed as one regulatory system by

which the normal cell is "protected" against hyperinsulinemia. This response would not play a physiologic role if the cell is resistant to the biological effects of insulin. The opposite response, "up regulation," might be expected. For example, glucocorticoids in vitro increase the number of insulin receptors in cultured rat hepatocytes (9), 3T3 mouse fibroblasts (38), and human lymphocytes (39). The rat hepatocytes rendered insulin resistant with glucocorticoids fail to down regulate in response to insulin (9). Also, the glucocorticoids-treated human lymphocytes demonstrated a rightward shift to the dose-response curve for down regulation by insulin (39). However, short-term administration of glucocorticoids in vivo is generally associated with decreased insulin binding (40). Interestingly, when glucocorticoids exposure is prolonged for 3 or 4 wk (9, 41), Olefsky et al. (41) observed partial recovery of insulin binding in hepatocytes and almost complete recovery in adipocytes. Caro and Amatruda (9) observed complete recovery of insulin binding in hepatocytes at the time that the cells were insulin resistant. It is possible, therefore, that different tissues in the same metabolic state or the same tissue in different metabolic state may respond differently to the ability of insulin to down regulate its receptor. In this regard it should be noted that insulin binding to monocytes (42) or erythrocytes (43) from uremic patients has been found to be normal (42) or decreased (42, 43)

The lack of linkage between insulin binding and insulin-stimulated AIB uptake led us to investigate insulin internalization (Fig. 2). It is presently well established that insulin enters the cell (44-46). However, the relationship of insulin internalization to the biological effect of insulin remains uncertain. It has been suggested that internalization of insulin may be necessary for inhibition of endogenous protein degradation (47), but not for insulin stimulation of glycogen synthetase activity (47) or amino acid transport (48). In the present work, it is apparent that the rate of insulin internalization is normal in the uremic and starving control animals (Figs. 2 and 3). Furthermore, since insulin binding is increased in these animals, they also have more intracellular insulin than ad lib.-fed controls. This finding demonstrated the lack of correlation between insulin internalization and insulin action with regard to AIB uptake.

After considerable controversy, the concept of a relationship between insulin binding and degradation has been well established (4, 10, 16). Thus, it might be expected that hepatocytes from uremic and starving control animals degrade more insulin than the normal animals because insulin binding is increased. However, although the relationship between insulin binding and degradation is maintained (Fig. 5), there is less degradation for a given amount of insulin bound. Thus, although insulin binding and degradation may be linked in normal metabolic states, it is apparent that the degradative system(s) following binding are also regulated by factors perhaps independent of the receptor complex. One such factor may be that which regulates the concentration of insulin receptor and mediates the biological effects of insulin. Two other insulin-resistant models that are hyperinsulinemic and do not have a decrease in hepatic insulin binding have normal rates of insulin degradation, i.e., obese Zucker rats (18) and glucocorticoid-treated rats (9).

It is not known whether insulin has to be internalized prior to degradation or if degradation can take place at or close to its binding site in the plasma membrane. The chemical method used in this study that discriminates between internalized and membrane-bound material allowed us, in freshly isolated hepatocytes, to examine by gel chromatography the nature of the radioactive material in membrane-bound and internalized components. Similar to our previous studies with primary cultures of normal rat hepatocytes (11), we have demonstrated that a significant portion of the final degradation products of insulin (peak III of the chromatograph) are found at both the membrane and the intracellular compartments (Table II). The slowed rate of insulin degradation and diminished quantity of iodinated insulin degradation products appearing in the medium from hepatocytes of uremic animals appears to be due mainly to a decrease in the activity of the membrane degradation system (Table II). The plasma membrane insulin degradation system may have an important function in the regulation of insulin biological activity and of the fraction of bound insulin available for internalization.

These studies emphasize the primary role of postreceptor events in the regulation of insulin binding, degradation, and action. Evidences for this includes hyperinsulinemic-resistant states with normal (31-37, 49)or high concentrations of insulin receptors (9, 50) and normal or low insulin degradation rates (9); the demonstration that cellular ATP may regulate insulin binding (51); the inability of insulin to down regulate the insulin receptor of turkey erythrocytes, a cell that lacks active macromolecular synthesis (52); the inability of insulin in vitro to down regulate the insulin receptor in insulin resistant cells (9, 53), and the ability of agents that mimic the effects of insulin without interacting with the insulin binding sites to regulate insulin binding and degradation (6).

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