Metabolic Characteristics of Renal Insulin Uptake

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SUMMARY

The kidney removes insulin from the circulation by glomerular and peritubular clearance, thus exposing both the luminal and the contraluminal tubular surfaces to the hormone. In the proximal tubule, filtered insulin is internalized by means of pinocytosis and eventually localizes in the lysosomes. The fate of insulin removed from the peritubular circulation is less well understood. We previously reported that tubular uptake of insulin is a temperature-sensitive process consisting of two different systems. The system associated with the luminal aspect of the cell appears to be dependent on oxidative metabolism, whereas the system associated with the contraluminal aspect of the cell appears to be independent thereof. To further characterize renal tubular insulin uptake, a study was performed with isolated perfused rat kidneys. In control experiments, total organ clearance of insulin (OCi) was 1336 ± 51 μl/min (SEM), glomerular filtration rate (GFR) was 1019 ± 54 μl/min, peritubular clearance of insulin was 317 ± 56 μl/min, fractional excretion of sodium (FEna) was 5.1 ± 0.7%, and fractional excretion of insulin (FEi) was 1.3 ± 0.6%. Addition of inhibitors of glycolysis (iodoacetate), Na-K-ATPase (ouabain), and lysosomal degradation (chloroquine) was associated with a significant increase of Fei (4.7 ± 0.4%, 6.4 ± 1.4%, and 13.9 ± 2.4%, respectively) and of FEna (28%, 43%, and 12%, respectively). By contrast acetazolamide with furosemide increased FEna to 39%, without altering FEi. Analysis of all data revealed no correlation between FEna and FEi, indicating that tubular insulin absorption is at least partly independent of sodium reabsorption. None of the inhibitors altered OCi, GFR, or peritubular clearance significantly.

The above observations, together with our previously reported data, indicate that luminal insulin uptake is a temperature and chloroquine sensitive process that is dependent on metabolic energy. The source of this energy appears to be ATP, which is derived from oxidative metabolism and glycolysis. These characteristics, consistent with a process involving pinocytosis and lysosomal degradation, were not features of the contraluminal insulin uptake process. DIABETES *30*:929–934, November 1981.

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With a better understanding of the metabolic derangements associated with renal failure, it has become apparent that the kidney plays a key role in the removal of low molecular weight (MW) proteins from the systemic circulation.[1] This process is achieved in the main by glomerular filtration followed by near complete tubular absorption.[1] In the case of insulin and several other small biologically active peptides, removal is also achieved by extraction from the peritubular vessels.[2–4] Filtered insulin is predominantly absorbed in the proximal tubule, although a small amount may be absorbed in the distal nephron.[5] Both in man and in the intact rat less than 1% of the filtered insulin is excreted in the urine.[2,3] Recently, by means of the isolated perfused rat kidney, we provided evidence suggesting that tubular cells take up insulin by two functionally and anatomically distinct pathways:[6] a luminal pathway for extracting insulin from the filtrate which is dependent on oxidative metabolism and a contraluminal pathway for extracting insulin from the peritubular compartment which is independent thereof. From electron microscopic autoradiographic studies by Bourdeau et al.,[7] it appears that luminal removal of filtered iodoinsulin occurs by means of pinocytosis with localization of the internalized protein in lysosomal-like bodies.

The purpose of the present study was to further characterize the metabolic requirements of renal tubular insulin uptake and to examine the participation of lysosomal degradation in the luminal and peritubular insulin uptake processes. The study was carried out with the isolated rat kidney per-

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fused with a continuously recirculating electrolyte-albumin solution, and this model has proved particularly useful in studying peptide-hormone metabolism.[1,4,6] The results of this study suggest that luminal insulin uptake has the metabolic characteristics of a pinocytotic process associated with lysosomal degradation, while peritubular uptake lacks these features.

METHODS

The study was performed with isolated kidneys obtained from male Sprague-Dawley rats weighing 290–390 g and allowed free access to food and water. Kidneys were isolated and perfused at 37°C as previously described.[4,6] After inducing anesthesia with 100 mg/kg intraperitoneal inactin (Promonta, Hamburg, West Germany), 50 mg/100 g mannitol was given intravenously. The abdomen was then opened by a midline incision and the right ureter was catheterized with polyethylene-10 tubing. Heparin (200 U) was then injected intravenously, and a glass cannula was inserted into the superior mesenteric artery and then into the aorta. Perfusate was allowed to flow through the cannula, which was inserted into the right renal artery. Then the kidney was rapidly excised and placed in a perfusion apparatus that incorporated 8-μm millipore filters in the circuit. Perfusate was recirculated with a Watson Marlow MHRE3 pulsatile flow pump (Watson Marlow Ltd., Cornwall, England) at an effective maximum pressure of either 100 or 120 mm/Hg distal to the tip of the cannula. The higher pressure was used in those experiments associated with a marked natriuresis in order to offset a fall in glomerular filtration rate (GFR).[9] The perfusate consisted of a modified Krebs-Henseleit solution containing 8 g of albumin per 100 ml (Fraction V, Miles Laboratories, Inc., Elkhart, Indiana) and was prepared as previously described.[4] To the perfusate was added glucose (5 mM), creatinine (4.4 mM), and a mixture of L-amino acids,[10] which included methionine (0.5 mM), alanine (2.0 mM), proline (2.0 mM), glycine (2.0 mM), isoleucine (2.0 mM), serine (2.0 mM), arginine-HCL (1.0 mM), and aspartic acid (3.0 mM). Before use, the perfusate was passed through an 0.8-μm filter and then a 0.45-μm millipore filter. Then it was continuously gassed with 95% O_2 and 5% CO_2, and the pH was adjusted to 7.4.

After perfusion of the kidney had begun, [^{14}C]inulin (Amersham/Searle Corp., Arlington Heights, Illinois) was added to the perfusate and a control perfusate sample obtained. At 9 min, highly purified porcine insulin (Lot #615-077-50, a gift of Dr. R. Chance of Eli Lilly and Co., Indianapolis, Indiana) was added. This produced an insulin concentration of 24 ± 5 ng/ml in the perfusate sample taken 6 min later. After allowing at least 15 min from the start of the perfusion for stabilization of renal function, five or six 10-min urine collections were obtained, and perfusate samples were taken at the beginning and end of each collection period. Urine samples were collected in albumin-coated tubes to prevent loss of insulin by adherence to glass. The perfusate volume at the start of urine collection was adjusted to 70 ml. Perfusate sample volumes (1 ml) were not replaced, but urine volumes were replaced with an appropriately hypotonic saline solution and evaporative losses with distilled water. In several experiments, perfusate samples were obtained at the end of perfusion and then incubated at 37°C for 60 min. Measured insulin concentrations before and after incubation were essentially the same, excluding the possibility of spontaneous degradation or degradation due to the release of enzymes.

Experimental groups. The kidneys were perfused under several different conditions. (1) Eight kidneys were perfused under standard conditions at a maximum effective pressure of 100 mm/Hg and served as controls. (2) Six kidneys were perfused at 120 mm/Hg with perfusate containing ouabain in a 4-mM concentration, which produces maximal depression of tubular sodium reabsorption in the isolated kidney by inhibiting sodium-potassium activated adenosine triphosphatase.[9] (3) Five kidneys were perfused at 120 mm/Hg with perfusate containing 0.1 mM iodoacetate, an inhibitor of glycolysis. (4) Five kidneys were perfused at 100 mm/Hg with perfusate containing 0.5 mM chloroquine, a lysosomotropic agent. (5) Five kidneys were perfused at 120 mm/Hg with 5 mM acetazolamide and 1 mM furosemide in the perfusate.

As preliminary studies revealed no significant difference between the uptake and excretion of insulin by kidneys perfused at 120–140 mm/Hg (N = 5) as compared with kidneys perfused at 100 mm/Hg (N = 8), this latter group served as controls for all the groups studied.

Analytic methods. Insulin was assayed in perfusate and urine samples by a modified double antibody radioimmunoassay method with reagents obtained from Cambridge Nuclear (Billerica, Massachusetts).[11] Standard curves of insulin in urine or in perfusate did not differ from standard curves of insulin in buffer. The between-assay coefficient of variation was 10% and the within-assay coefficient of variation was 2%. Sodium was measured with a Beckman KLINA flame photometer (Beckman Instruments, Inc., Fullerton, California) and [^{14}C]inulin was counted in a Searle Mark II liquid scintillating counter (Nuclear Chicago, Des Plaines, Illinois).

Calculations. In each experiment the organ clearance of insulin (OCi), which represents the volume of perfusate from which the kidney completely and irreversibly removes insulin each minute, was calculated between successive 10-min samples according to Mortimore et al.:[12] $OCi = 2.3\ V/\Delta t \log [(C_1 - C\alpha)/(C_2 - C\alpha)]$, where V = volume of perfusion fluid corrected for perfusate sampling; C_1 and C_2 = initial and final concentrations over time interval t; and $C\alpha$ = asymptote approached by C after prolonged perfusion. $C\alpha$ was taken as zero because prolonged perfusion at low concentrations of insulin indicated the eventual disappearance of the protein. We have previously validated the use of this formula[26] which was confirmed in this study by finding that the clearance of [^{14}C]inulin determined by this method was similar to that determined by the standard urine clearance formula described below. In the control experiments these clearance values were 1005 ± 65 and 1019 ± 54 μl/min, respectively, $P > 0.05$, using Student's paired *t* test. The kidney clears insulin from the circulation by means of filtration (i.e., glomerular clearance, and by extraction from the peritubular vessels, i.e., peritibular clearance.) Assuming there is little hindrance to the filtration of insulin at the glomerulus and that none of the filtered hormone is reabsorbed intact into the peritubular circulation, the glomerular clearance of insulin was taken to equal the glomerular filtration rate. The peritubular clearance was taken to equal the difference between the organ clearance and the glomerular

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clearance of insulin. However, this calculation would probably underestimate peritubular clearance for it is unlikely that insulin is freely filtered.[6] The urinary clearance of insulin and of [^{14}C]inulin were calculated from the urine flow and the urine:plasma concentration ratios. The clearance of [^{14}C]inulin was used as a measure of GFR. Fractional excretion of insulin (FEi) was calculated as the urinary insulin clearance/GFR × 100 and fractional excretion of filtered sodium (FEna) as sodium clearance/GFR × 100. Perfusion flow was determined from a Brooks Flow Meter inserted into the perfusion circuit (Emerson Rotameter, Brooks Instruments, Hatfield, Pennsylvania).

The average of all the measured values in each experiment was taken as the value for that experiment. Results are expressed as the mean ± SEM. The data were statistically analyzed with a one-way analysis of variance. If the F ratio was significant, Dunnetts test was used to make comparisons between the control and the experimental means.[13] When required to correct for nonhomogeneity of variances, a square root transformation of data was used. A level of $P < 0.05$ was regarded as significant.

RESULTS

The perfusate was modified by adding amino acids and creatinine according to the description of DeMello and Maack.[10] This was associated with a higher GFR, 1019 ± 54 μl/min, and a lower FEi, 1.3 ± 0.6%, than we have previously reported.[6]

The organ clearance of insulin (Table 1). In the control kidneys the OCi determined from its rate of disappearance from the perfusate averaged 1336 ± 51 μl/min. The addition of iodoacetate to the perfusate was not associated with a significant change in OCi, 1295 ± 115 μl/min, when compared with the controls. Although the mean OCi in the experiments with ouabain (1167 ± 107 μl/min), chloroquine (1100 ± 31 μl/min), and diuretics (1171 ± 48 μl/min) was lower than the mean of the control experiments, there was no significant difference between the treatment groups and the controls.

Glomerular clearance of insulin (assumed to equal GFR). Table 1 summarizes the GFR in the various groups studied. There was no significant difference between the GFR of the treatment groups and that of the controls, 1019 ± 54 μl/min. This suggests that the amount of insulin removed by filtration, i.e., glomerular clearance, was similar under the different conditions studied. Assuming that insulin passes through the glomerular filtration barrier with relatively little restriction and that the GFR is taken as a measure of the glomerular clearance of insulin, then the glomerular clearance in the controls could account for only 76.6 ± 3.8% of the OCi. Similar findings were obtained in the other groups studied (Table 1).

Peritubular clearance of insulin (Table 1). The glomerular clearance of insulin could not account for all the insulin removed, and it is presumed that the balance was removed by uptake from the peritubular circulation (i.e., peritubular clearance). In the controls, the peritubular clearance was 317 ± 56 μl/min and accounted for 23.4 ± 3.8% of the OCi; similar values were observed in the treatment groups.

Fractional excretion of insulin and sodium (Table 1). In the control kidneys, fractional excretion of insulin was 1.3 ± 0.6% and fractional excretion of filtered sodium was 5.1 ± 0.7%. Addition of iodoacetate and ouabain to the perfusate produced a significant increase in the fractional excretion of insulin (4.7 ± 0.4% and 6.4 ± 1.4%, respectively) and the fractional excretion of filtered sodium (27.6 ± 2.7% and 43.1 ± 1.4%, respectively) as compared with the controls. When kidneys were perfused with chloroquine, fractional insulin excretion (13.9 ± 2.6%) and fractional sodium excretion (12.0 ± 2.6%) also increased significantly when com-

TABLE 1
The uptake and excretion of insulin by the isolated rat kidney perfused under different conditions*

	Control	Iodoacetate (0.01 mM)	Ouabain (4 mM)	Chloroquine (0.5 mM)	Diamox (5 mM) + furosemide (1mM)
No. of experiments	8	5	6	5	5
Effective maximum perfusion pressure (mm/HG)	100	120	120	100	120
OCi (μl/min)	1336	1295	1167	1100	1171
	±51	±115	±107	±31	±48
GFR = GCi (μl/min)	1019	1023	846	815	893
	±54	±74	±57	±52	±52
PCi (μl/min)	317	272	321	285	278
	±56	±105	±111	±52	±55
UCi (μl/min)	12	46†	50†	108†	21
	±6.1	±3.4	±8.0	±18	±8.8
GC/OCi (%)	76.6	78.9	75.2	72.7	76.5
	±3.8	±6.5	±8.0	±5.2	±4.5
PC/OCi (%)	23.4	21.1	24.8	27.3	23.5
	±3.8	±6.5	±8.0	±5.2	±4.5
UC/GCi = FEi (%)	1.3	4.7‡	6.4†	13.9†	2.2
	±0.6	±0.4	±1.4	±2.4	±1.3
FEna (%)	5.1	27.6†	43.1†	12.0†	38.7†
	±0.7	±2.7	±1.4	±2.6	±2.3
RPF (ml/min)	50	47	37†	50	44
	±3.0	±2.7	±1.1	±1.8	±1.6

* Values are means ± SEM. † $P < 0.01$ vs. control. ‡ $P < 0.5$ vs. control. Abbreviations: OCi, organ clearance of insulin; GFR, glomerular filtration rate; GCi, glomerular clearance of insulin; PCi, peritubular clearance of insulin; UCi, urinary clearance of insulin; FEi, fractional excretion of insulin; FEna, fractional excretion of sodium; RPF, renal perfusion flow.

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pared with the controls. By contrast, the addition of both acetazolamide and furosemide to the perfusate was associated with a significant increase in the fractional excretion of filtered sodium (38.7 ± 2.3%) without a significant alteration in fractional excretion of insulin (2.2 ± 1.3%). Regression analysis of the data from all the experiments showed no significant correlation between the fractional excretion of insulin and fractional excretion of filtered sodium (Figure 1).

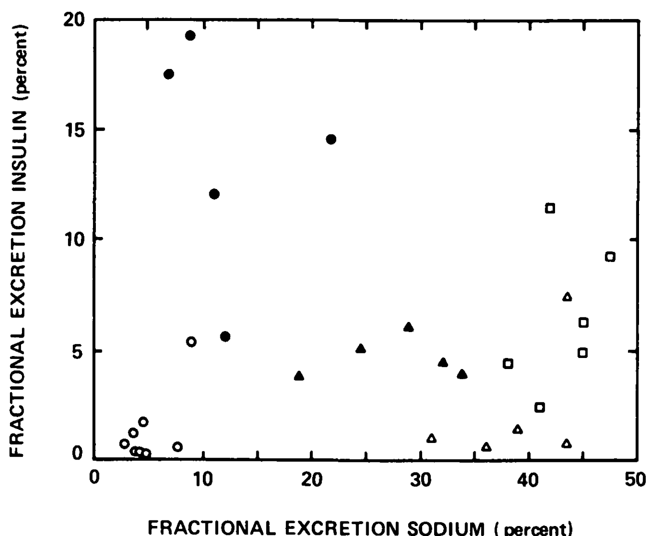
Renal perfusion flow (Table 1). Renal perfusion flow averaged 50 ± 3 ml/min in the controls. Addition of ouabain to the perfusate was associated with a significant fall in perfusion flow, 37 ± 1 ml/min. In the other treatment groups, there were no significant differences in perfusion flow as compared with the controls.

DISCUSSION

The kidney is a major site of insulin metabolism, clearing the hormone from the circulation by means of two pathways, glomerular and peritubular clearance.[2,3,6] Knowledge of the process whereby the tubular cells remove insulin from the tubular lumen and from the peritubular compartment is essential to an understanding of the renal metabolism of insulin.

It is well established from electron microscopic autoradiography that filtered insulin, like other proteins, is internalized in the proximal tubule by means of pinocytosis,[7,14] a process that may be activated by the binding of insulin to the brush border membrane.[15] Following internalization, the filtered insulin eventually localizes in the lysosomes[7] where digestion of proteins occurs.[14] Several features of this filtration-absorption-degradation pathway are incompletely understood, including the nature of the metabolic requirements of the pinocytotic process and the role of lysosomes in the digestion of individual proteins. According to a recent review by Silverstein et al.,[16] the metabolic characteristics of pinocytosis in extra renal tissues include a sensitivity to temperature and a dependence on metabolic energy (probably ATP) which may be derived from either glycolytic or oxidative metabolism. Whether these features characterize the process whereby a protein, such as insulin, is internalized in the kidney is the subject of the present study. Regarding the role of lysosomes in renal insulin degradation,[17] it has been generally accepted that these subcellular bodies are the main site of protein digestion.[1,14] However, as other subcellular structures in the tubular epithelium also possess insulin-degrading properties,[18,19] the role of lysosomes in renal insulin degradation is uncertain and is examined in this study. Note that in intact adipocytes[20,21] and hepatocytes,[22] both lysosomal and nonlysosomal degradation occurs. In adipocytes lysosomal degradation plays a minor role, whereas in hepatocytes it may account for up to 50% of cellular insulin degradation.

FIGURE 1. Relationship between fractional excretion of insulin and fractional excretion of filtered sodium in isolated rat kidneys perfused under different conditions (○ controls, ● chloroquine 0.5 mM, □ ouabain 4mM, ▲ iodoacetate 0.1 mM, △ acetazolamide 5 mM with furosemide 1 mM). No significant correlation is evident.



In contrast to the relatively well-defined filtration-absorption pathway, it is unclear whether internalization and lysosomal degradation of insulin removed from the peritubular circulation occurs, for pinocytosis is not a prominent feature of this aspect of the tubular cell. From recent studies it appears that peritubular uptake involves the binding of insulin to specific receptors,[19,23,24] a process followed by degradation[19,24,25] and perhaps initiation of the renotropic actions of the hormone.[26] It is unclear whether degradation occurs while insulin is bound to the cell membrane or after it is internalized. Apart from these differences between the luminal and peritubular uptake processes, we have previously reported[6] that although both processes are temperature sensitive, they differ functionally because luminal uptake is dependent on oxidative metabolism whereas peritubular uptake is independent.

This study using the isolated perfused rat kidney examines the metabolic requirements of tubular insulin uptake, and the participation of lysosomal degradation in the luminal and peritubular insulin uptake process. In the control kidneys the GFR averaged 77% of the OCi; thus peritubular clearance accounted for at least 23% of the insulin extracted. These values differ from the value obtained from the intact rat (where the renal organ clearance of insulin is similar to GFR), and peritubular clearance can only be demonstrated after reducing or abolishing GFR by obstructing urine flow.[3] Luminal uptake of insulin as judged by its fractional excretion, 1.3%, was similar to that described in the intact rat.[3] It is apparent from these observations that caution must be exercised when extrapolating quantitative data obtained from the isolated kidney to the situation in the intact animal. A further limitation of the isolated perfused rat kidney model is that it does not permit the precise anatomical localization of metabolic events given the heterogeneity of renal tubular epithelium. Nevertheless, this model has been particularly useful in studying peptide hormone metabolism for it permits the use of maneuvers that are impractical to perform in the intact animal.[1,4,6]

Previously, we reported that fractional excretion of insulin in the isolated kidney perfused with a fuel-free perfusate was similar to that of kidneys perfused with a glucose-containing perfusate.[6] This was interpreted as indicating that glucose is not required as an energy source for renal tubular insulin transport. However, as the kidney has the capacity to produce glucose, the absence of an exogenous supply does not preclude the possibility that endogenous glucose may serve as an energy source for tubular protein uptake.

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Therefore, in the present study, kidneys were perfused with iodoacetate in a concentration known to produce a marked decrease in glycolysis and only relatively small decreases in other metabolic energy-producing processes in the isolated kidney.[27] Use of this inhibitor increased the fractional excretion of insulin to a value threefold greater than in the control experiments, indicating that glycolysis may indeed serve as an energy source for insulin absorption. Peritubular insulin clearance was essentially unaltered by the presence of iodoacetate. The contribution of glycolysis to energy production in the cortex is small, whereas in the medulla its contribution is large,[28] and it is conceivable that inhibition of glycolysis may have affected the small fraction of insulin absorbed in the distal nephron[5] rather than the larger fraction absorbed in the proximal tubule. The possibility also exists that iodoacetate may have increased insulin excretion by nonspecific means.

Perfusion of kidneys with ouabain in a concentration that produces maximal depression of tubular sodium reabsorption[9] produced a fivefold increase in the fractional excretion of insulin as compared with controls. Peritubular insulin clearance remained essentially unaltered. In a study with rat kidney slices, Miller et al.[29] noted that ouabain inhibited the luminal uptake of horseradish peroxidase, a protein of molecular weight 40,000 daltons. These studies suggest that hydrolysis of ATP is required for energizing the internalization of filtered proteins. In contrast to our findings with insulin, we previously reported that ouabain did not alter the fractional excretion of arginine vasopressin in the isolated perfused rat kidney.[4] This difference could be explained if the nonapeptide arginine vasopressin is not absorbed in the intact form, but instead undergoes enzymatic hydrolysis on the surface of the brush border with absorption or excretion of the end products. Although it appears likely that ATP may serve as an energy source for internalizing the filtered insulin, and that perfusion with ouabain or iodoacetate impairs internalization directly by interfering with this energy source, it must also be recognized that intracellular protein degradation has a requirement for energy in the form of ATP.[30] It is possible that ouabain and iodoacetate may impair insulin absorption indirectly by compromising insulin degradation. However, in adipocytes[20,33] and hepatocytes,[22] agents that deplete the cell of ATP block the delivery of insulin to the lysosomes. Impaired solute transport is another potential mechanism, whereby interference with ATP production might indirectly impair insulin absorption. This is discussed later.

Chloroquine is an agent that is rapidly taken up by cells and then selectively concentrated in the lysosomes where it inactivates protein degradation.[31,32] The mechanism of action is not entirely clear but may be due to an alteration in intralysosomal pH or by direct inhibition of enzyme activity. Several studies carried out with isolated adipocytes[20,21,33] and hepatocytes[22] have shown that chloroquine is effective in inhibiting lysosomal insulin degradation. In order to assess the participation of lysosomal degradation in renal insulin metabolism, we perfused isolated kidneys with a chloroquine-containing perfusate. It was reasoned that if insulin is degraded by a chloroquine-sensitive lysosomal system then perfusion with chloroquine should impair renal insulin uptake. Indeed chloroquine was associated with a marked impairment in tubular absorption of insulin; fractional excretion of insulin was 10-fold greater than in the controls. Although it is possible that this effect might be secondary to a direct action on the pinocytotic process rather than to inhibition of degradation, studies in isolated fat cells[20,21,33] and hepatocytes[22] have failed to substantiate this possibility. In contrast to luminal uptake, chloroquine had no significant effect on peritubular uptake. This suggests that lysosomal degradation is not involved in the peritubular removal process. That chloroquine only partly impaired the tubular absorption of insulin may reflect the extent of lysosomal inhibition achieved or may be indicative of the presence of nonlysosomal pathways for the degradation of absorbed insulin.

As both insulin and sodium transport were impaired by the various maneuvers employed, we set out to evaluate the relationship between these two transport processes by perfusing isolated kidneys with acetazolamide and furosemide. These agents used in high concentrations inhibit proximal tubular reabsorption of sodium,[34] although the major effect of furosemide is on the loop of Henle. Despite a marked increase in the fractional excretion of filtered sodium to 39%, fractional excretion of insulin did not alter significantly. These findings suggest that tubular insulin absorption is at least in part independent of sodium reabsorption. Analysis of the data from all the groups including the controls revealed a lack of correlation between the fractional excretion of insulin and fractional excretion of filtered sodium (Figure 1). This does not, however, exclude a possible link between insulin and sodium transport. Whereas fractional excretion of insulin reflects mainly a proximal tubular function, changes in fractional excretion of sodium may reflect the action of an inhibitor on several nephron segments.

Previously, we reported that renal tubular epithelial cells appear to possess two functionally and anatomically distinct systems for the uptake of insulin.[6] Both systems are temperature sensitive, but the luminal system that removes filtered insulin is dependent on oxidative metabolism, whereas the peritubular system that removes insulin from the peritubular compartment is independent. In the present study, further differences were observed. Luminal uptake was significantly depressed by the presence of inhibitors of ATP production or hydrolysis and by an inhibitor of lysosomal function. These findings were consistent with a process involving pinocytosis and lysosomal degradation. By contrast, peritubular uptake did not possess these features; these important negative findings further indicate that the peritubular system (for the removal and degradation of insulin) differs from the luminal system.

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