Evidence for a Direct Action of Insulin to Increase Renal Reabsorption of Calcium and for an Irreversible Defect in Renal Ability to Conserve Calcium Due to Prolonged Absence of Insulin

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SUMMARY

Male rats were injected with streptozotocin (STZ), 60 mg/kg, i.p., on 2 successive days. Six hours after the last STZ injection, some STZ-diabetic rats began receiving daily injections of insulin that were insufficient to control blood glucose. Another group of STZ-diabetic rats received insulin injections after 2 wk duration of untreated diabetes. Still other STZ-diabetic rats received no insulin treatment. Under sodium pentobarbital anesthesia, kidneys from each treatment group were isolated and perfused with an artificial "plasma" containing ^{45}Ca. As urine was collected, urine-to-perfusate ultrafiltrate (U/P) ratios for ^{45}Ca were determined. The results of the studies showed that: STZ diabetes reduced ^{45}Ca reabsorption by the kidney; the increased urinary excretion of calcium was not due to an osmotic effect or to a direct nephrotoxic action of STZ; and insulin therapy instituted early, but insufficient to control blood glucose, reduced the diabetes-induced calcium loss via a direct action on the kidney, whereas insulin therapy instituted late failed to reverse renal loss of calcium. DIABETES 1984; 33:991–94.

The importance of the calcium ion in the maintenance of normal functioning of nerves and vascular smooth muscle, together with evidence linking insulin's action to the calcium ion,[1-5] prompted us to speculate that in a disease of insulin deficiency, i.e., diabetes mellitus, calcium metabolism might be altered and that such alterations could result in neuropathies and microangiopathies, the long-term complications of diabetes. Thus, in earlier studies, we reported that ^{45}Ca uptake by slices of kidney cortex from STZ-diabetic rats[6] and from alloxan-diabetic rats[7] was significantly below control values. Furthermore, we found that daily insulin treatment instituted immediately upon production of both models of diabetes prevented the diabetes-induced depression of ^{45}Ca uptake even though the insulin treatment was insufficient to control blood glucose. We reported that when insulin therapy was delayed for a period of 1 mo, it was ineffective in restoring ^{45}Ca uptake to normal levels in slices of kidney cortex from these alloxan- or STZ-diabetic rats even when blood glucose was controlled. Finally, we showed in both of these studies that when insulin was added in vitro to slices of kidney cortex from control kidneys, an increase in ^{45}Ca uptake by the slices was noted.

The present studies were designed to allow for direct control of glucose concentrations delivered to perfusing kidneys to further test our hypothesis that renal calcium transport is altered by the diabetic state and that daily insulin treatment instituted early will prevent this alteration by a direct action on the kidney that is unrelated to its effect on blood glucose.

MATERIALS AND METHODS

Animals and treatments. Age-matched, male, Sprague-Dawley rats with initial body weights of approximately 250 g were used throughout all experiments. The rats were divided into four main treatment groups: controls, streptozotocin-diabetic (STZ), early insulin-treated STZ-diabetic (IE), and delayed insulin-treated STZ-diabetic (ID). To produce diabetes, animals were injected i.p. on 2 successive days with 60 mg/kg STZ, freshly dissolved in citrate buffer, pH 4.5. The IE group began receiving 1.5 U of insulin (NPH) daily (s.c.) starting within 6 h of the second STZ injection. The ID group began receiving insulin as above starting 2 wk after the second STZ injection. The dose of NPH used has previously been shown to be insufficient for control of blood glucose.[6-9] Rats were maintained ad libitum on standard laboratory rodent chow and tap water and were housed in a well-ventilated, temperature-controlled animal room with automatic 12-h light/dark cycles.

Drugs and chemicals. ^{45}Ca, 1 mCi (initial sp act = 20 mCi/mg) was obtained from New England Nuclear Corp., Boston,

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Massachusetts. It was diluted with 0.9% saline for use in these studies. Streptozotocin (STZ) was obtained from Sigma Chemical Co., St. Louis, Missouri. NPH (U-100, Iletin I, Isophane insulin suspension) was obtained from Eli Lilly and Company, Indianapolis, Indiana, and was diluted in 0.1 M phosphate buffer, pH 7.4, to give a final concentration of 10 U/ml. Heparin, 1000 U/ml, was obtained from Organon Inc., West Orange, New Jersey. Bovine albumin, fraction V, was obtained from Miles Laboratories, Elkhart, Indiana. Other chemicals and reagents of analytic grade were obtained from commercial suppliers.

Experimental procedures. Since a maximum of two kidney perfusions could be performed in 1 day, inductions of STZ-diabetes and times for initiation of insulin treatment were staggered. This made it possible to keep durations of the conditions the same for several animals whose kidneys were to be isolated and perfused on different days. Kidneys from age-matched control rats were always perfused on the same days with those from the other treatment groups. After 2 wk duration of untreated STZ diabetes (STZ group), 2 wk duration of NPH-treated STZ diabetes (IE group), and 2 wk duration of untreated diabetes followed by 2 wk duration of NPH-treated diabetes (ID group), kidneys from age-matched control rats and from the above treatment groups were isolated and perfused according to the procedure that follows. Under sodium pentobarbital anesthesia (60 mg/kg, i.p.), the peritoneal cavity was opened and blood glucose concentrations were semiquantitatively measured by touching drops of fresh blood with Chemstrip bG test strips (Bio-Dynamics/bmc, Indianapolis, Indiana). The right kidney, its ureter and renal artery, the aorta, inferior vena cava, and superior mesenteric artery were carefully isolated from surrounding fat and tissue. A PE-10 catheter was placed in the right ureter and secured with a tie. After injection of heparin (500 U) into the inferior vena cava, a 22-guage × 1-in arterial catheter (Abbocath-T) was inserted into the superior mesenteric artery, threaded through the aorta into the right renal artery, and secured in place with ties. Perfusion was begun, and immediately thereafter the aorta above the renal artery was ligated and cut, as was the inferior vena cava. The intact perfused kidney was transferred to a specially constructed, plexiglass-enclosed, temperature-controlled (37 ± 1°C) perfusion apparatus. Perfusion medium was continuously recirculated at a flow rate of 25–30 ml/min and at a pulsatile pressure of 120/90 mm Hg distal to the tip of the arterial cannula. The standard perfusion medium was prepared as described by Ross et al.[10] and contained, in mmol/L: sodium, 143; potassium, 5.35; calcium, 1.25; magnesium, 1.18; bicarbonate, 25; chloride, 123; phosphate, 1.2; sulfate, 0.77; and 6–7% bovine serum albumin. The medium was continuously gassed with 95% O_2/5% CO_2. Glucose was added to the standard perfusion medium in quantities to give final concentrations representing normoglycemia (100 mg/dl) for perfusion of kidneys from control rats, and hyperglycemia (300 mg/dl) for perfusion of kidneys from NPH-treated and untreated diabetic rats as well as for perfusion of kidneys from control rats. When insulin was added, 1 U of NPH was added to 100 ml of the medium. After 15 min of perfusion, when urine flow was approximately 20 μl/min, ^{45}Ca ($6–7 \times 10^6$ cpm) was added to the perfusate vessel through a side arm and samples of urine from the cannulated ureter and of the perfusate were taken at 15-min intervals over a period of 90 min.

Ultrafilterable ^{45}Ca was obtained by placing samples of perfusate in dialysis bags, which, in turn, were placed in centrifuge tubes and supported by a perforated plexiglass dish. The tubes were gassed to maintain pH and were then centrifuged at 3000 × *g* for 1 h. This procedure essentially follows that described by Toribara et al.[11] Glomerular filtration rates were determined via ^{14}C-inulin clearance measurements as described by Sullivan.[12] Specific activities of ^{45}Ca in urine and ultrafiltrates of the perfusion medium were determined using atomic absorption spectrometry according to the method of Savory et al.[13] and liquid scintillation spectrometry. Statistical analyses of data were performed using Student's *t*-test and analysis of variance. Means were considered significantly different when $P < 0.05$. All radioactivity measured was corrected for efficiency of the counter and for quenching. STZ diabetes was confirmed using Bili-Labstix (Ames) and finding persistent (i.e., daily) glucosuria exceeding 500 mg/dl.

RESULTS

All diabetic rats, i.e., both the untreated and NPH-treated, had glucosuria exceeding 500 mg/dl and there were, throughout all studies, no differences between the semiquantitative glucose concentrations in urines from untreated (from 1 to 2 g/dl) and NPH-treated (from 1 to 2 g/dl) rats nor were there any significant differences in blood glucose values between these two groups. Blood glucose concentrations (at the time of surgery), in mg/dl, were as follows: control, 114 ± 10; STZ, 775 ± 15; IE, 748 ± 17; and ID, 760 ± 12. At no time in any of the studies was there evidence of proteinuria.

Specific activities of ^{45}Ca in urine and ultrafiltrates were equal within 10 min of the addition of ^{45}Ca to the perfusate. Glomerular filtration rates of kidneys from the four treatment groups are given in Table 1. Filtration rates were higher in kidneys from all diabetic rats and were highest in kidneys from the animals whose insulin treatment was initiated after 2 wk duration of untreated diabetes.

Figure 1 summarizes data resulting from perfusions of kidneys isolated from control rats, untreated diabetic (STZ) rats, rats with 2 wk duration of diabetes that began receiving daily

TABLE 1
Glomerular filtration rates of perfused kidneys

Treatment group	[Glu]*	[I]†	GFR (ml/min)
Cont	100	0	1.05 ± 0.34
Cont	300	0	2.50 ± 0.18‡
Cont	100	1.0	1.25 ± 0.23
Cont	300	1.0	2.39 ± 0.16‡
STZ	300	0	2.70 ± 0.37‡
IE	300	0	2.30 ± 0.36‡
ID	300	0	5.70 ± 0.30‡,§

*Glucose concentration of perfusate (mg/dl).
†Insulin concentration of perfusate (U/dl).
‡Significant difference from first control group.
§Significant difference from other diabetic groups.

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insulin therapy within 6 h of the last injection of STZ (IE group), and those with 2 wk duration of untreated diabetes followed by 2 wk duration of NPH-treated diabetes (ID group). The data show that kidneys from control rats do reabsorb calcium, in that the urine-to-perfusate ultrafiltrate (U/P) ratios of ^{45}Ca were <1.0. Kidneys from control rats gave identical U/P ratios of ^{45}Ca when the perfusate contained glucose in concentrations of 100 mg/dl and 300 mg/dl. Kidneys from STZ animals, as well as those from the ID group, did not actively reabsorb calcium, as evidenced by U/P ratios that were around 1.0. The kidneys from IE rats, whose daily NPH treatment had been of the same duration as had been the diabetic state, yielded U/P ratios of ^{45}Ca that were always less than unity and were not significantly different from U/P ratios of ^{45}Ca from control kidneys. Finally, the figure shows that when kidneys from control rats were perfused with media containing 1 U NPH/dl, U/P ratios of ^{45}Ca were significantly less than those obtained from control kidneys in the absence of insulin.

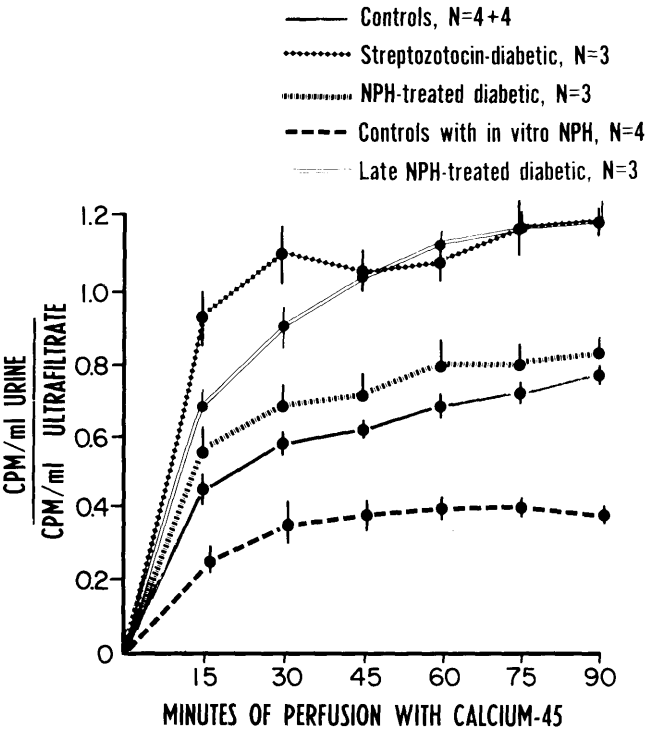

FIGURE 1. Urine-to-perfusate ultrafiltrate ratios of ^{45}Ca for kidneys from rats with different conditions as described in the text. N = number of kidneys perfused (= number of rats studied). Control N = 4 + 4 indicates 4 kidneys perfused with medium containing 100 mg glucose/dl and 4 perfused with medium containing 300 mg glucose/dl. The ratios for these control kidneys were identical regardless of the glucose concentration in the perfusate. Data are presented as means ± SEM.

DISCUSSION

Results of the present studies provide several lines of evidence for an effect of insulin on calcium metabolism that is independent of its effect on glucose metabolism. First, when kidneys from untreated diabetic rats were perfused with media containing 300 mg/dl glucose and ^{45}Ca, much more ^{45}Ca appeared in urine than appeared when kidneys from insulin-treated diabetic rats were perfused with identical perfusates. Second, reabsorption of ^{45}Ca by kidneys from control rats was the same when the media contained 100 or 300 mg/dl glucose. Third, at glucose concentrations in perfusate that were below the renal threshhold for glucose (100 mg/dl), addition of insulin to the perfusate resulted in increased reabsorption of calcium. The data in the present study directly support our hypothesis that insulin has a direct action on the kidney, since perfusion of kidneys from control rats with perfusate containing ^{45}Ca and 1.0 U insulin resulted in significantly decreased ^{45}Ca in urine when compared with kidneys from control rats that were perfused with insulin-free perfusate.

Specific binding sites for insulin with characteristics of receptors have been demonstrated throughout the nephron.[14-17] We suggest, therefore, that prolonged absence of insulin results in irreversible alterations in the insulin "receptors" such that the normal passive diffusion of calcium into renal cells from the lumen[5,18] is impeded. This, we believe, accounts for at least part of the negative calcium balance of diabetes.

We have recently reported on studies of the activities of Ca^{2+} and Mg^{2+} ATPases in kidneys of rats belonging to the same experimental groups as described in the present report.[18] We found increased activity of Ca^{2+}-ATPase in kidneys of diabetic rats. This increased activity on the contraluminal membrane of kidney cells, we believe, represents a futile attempt to conserve calcium in the face of depressed passive diffusion of calcium from urine into tubular cells.

Finally, we saw no correlation between glucose concentration in the perfusate and GFR values; nor was there evidence of a direct effect of insulin on GFR. It would seem, however, that the presence of insulin indirectly alters GFR, since delayed insulin treatment was the only difference between the STZ group and the ID group of rats and GFR values for kidneys from the ID group were approximately twice those of other kidneys being perfused with 300 mg/dl glucose. It is possible that the low dose of insulin used in our studies to sustain severe hyperglycemia, but control urinary calcium excretion, may have altered glomerular hemodynamics by some means independent of an effect of blood glucose.

In view of the absolute necessity for calcium in the correct functioning of cells,[19] the increased loss of calcium via the kidneys due to a diabetes-induced renal defect could account, in part, for the long-term complications of the diabetic state (e.g., neuropathies and microangiopathies affecting the eyes, heart, kidneys, and extremities). Our studies indicate that insulin's presence is required at functionally important sites, i.e., "receptors" in the kidney, to maintain sufficient calcium in the body. Although our studies indicate that this aspect of insulin's action appears to be independent of its effect on glucose transport, studies in human beings[20] indicate that control of blood glucose by insulin also controls urinary calcium excretion.

Although we have no direct evidence that loss of calcium is responsible for the long-term complications of diabetes mellitus, the possibility of a connection between the two should not be ignored. Should future investigations *prove* a causal relationship between the two, the first and essential step toward prevention of these complications would be made.

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