1,25-Dihydroxyvitamin D_3 and 24,25-Dihydroxyvitamin D_3 Production by Isolated Renal Slices is Modulated by Diabetes and Insulin in the Rat

NIRANDON WONGSURAWAT, H. JAMES ARMBRECHT, TERRY V. ZENSER, BERNARD B. DAVIS, MARY L. THOMAS, AND LEONARD R. FORTE

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SUMMARY

The alterations of mineral homeostasis observed in the streptozotocin-induced diabetic rat have been attributed to low circulating levels of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3], the biologically active metabolite of vitamin D_3. However, the effect of diabetes and subsequent insulin repletion on production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 by the kidney has not been investigated. We studied the renal production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 in diabetic and insulin-treated diabetic rats using the renal slice technique. In rats made diabetic with streptozotocin, the renal production of 1,25-$(OH)_2$-D_3 was markedly reduced (0.195 ± 0.011 pg/min/mg) compared with controls (0.685 ± 0.107 pg/min/mg, $P < 0.05$). The renal production of 24,25-$(OH)_2$-D_3 in diabetic rats was markedly increased (0.529 ± 0.052 pg/min/mg) compared with controls (0.233 ± 0.035 pg/min/mg, $P < 0.05$). Treatment of diabetic rats with insulin for 12 days resulted in a significant increase in 1,25-$(OH)_2$-D_3 production (0.331 ± 0.053 pg/min/mg) and decrease in 24,25-$(OH)_2$-D_3 production (0.329 ± 0.054 pg/min/mg). The possibility that diabetes decreased renal 1,25-$(OH)_2$-D_3 production by decreasing parathyroid hormone (PTH) secretion or depressing the action of PTH on the kidney was also studied. Diabetes caused no decrease in serum PTH levels relative to the control group and urinary cyclic AMP excretion in the diabetic group was not depressed. The cyclic AMP content of diabetic rat kidney slices in response to PTH was similar to controls (39.8 ± 3.3 versus 39.7 ±4.6 pmol/mg wet weight). Serum immunoreactive calcitonin (iCT) was significantly depressed in diabetic rats compared with controls. Insulin treatment of diabetic rats resulted in a marked increase in serum iCT. These data suggest the impairment of renal 1,25-$(OH)_2$-D_3 production was not related to impairment of the PTH activation of adenylate cyclase in the diabetic state. **DIABETES *32*:302–306, April 1983.**

From the Geriatric Center, VA Medical Center and the Departments of Medicine and Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri; and the VA Medical Center and Department of Pharmacology, University of Missouri, Columbia, Missouri.

Address reprint requests to Nirandon Wongsurawat, M.D., VA Medical Center (111JB), St. Louis, Missouri 63125.

Received for publication 30 August 1982.

Rats rendered diabetic by alloxan or streptozotocin injection demonstrate decreased intestinal calcium transport and calcium-binding protein.[1] In addition, plasma 1,25-$(OH)_2$-D_3 values are low.[2–4] Treatment with insulin results in normalization of plasma 1,25-$(OH)_2$-D_3 levels and correction of intestinal calcium malabsorption.[2–5] Administration of 1,25-$(OH)_2$-D_3 but not 25-OH-D_3 to diabetic rats corrects the depressed duodenal absorption of calcium.[6] The catabolism of 1,25-$(OH)_2$-D_3 in diabetic rats is not accelerated.[7] This suggests that there is a defect in the production of 1,25-$(OH)_2$-D_3 in diabetes. However, the site of the defect has not been unambiguously demonstrated since there is evidence that tissues other than the kidney also make 1,25-$(OH)_2$-D_3.[8] With the development of the renal slice technique,[9] it is possible to examine metabolism of 25-OH-D_3 by the mammalian kidney uncomplicated by further metabolism of the products by other organs. Therefore, we have used the renal slice technique to study the renal production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 in diabetic and insulin-treated diabetic rats. In addition, we have examined the status of calcitropic hormones considered to be modulators of renal 25-OH-D_3 metabolism in diabetic rats by measuring serum immunoreactive PTH (iPTH) and immunoreactive calcitonin (iCT) levels. It has been proposed that the decreased circulating 1,25-$(OH)_2$-D_3 in diabetes is related to decreased sensitivity of the diabetic kidney to the stimulation by PTH in vivo.[10] We, therefore, have also measured the effect of PTH on cyclic AMP production by diabetic rat kidney.

MATERIALS AND METHODS

Male F344 rats 5 wk of age (Charles River Breeding Laboratories, Wilmington, Massachusetts) were fed a diet containing 3 IU vitamin D/g diet, 0.02% calcium, and 0.6% phos-

phorus (Teklad Test Diet No. 78204, Teklad Test Diets, Madison, Wisconsin), and deionized water ad libitum for an additional 4 wk. Rats to be rendered diabetic received a single i.p. injection of 65 mg/kg streptozotocin (Sigma Chemical Co., St. Louis, Missouri) freshly dissolved in citrate buffer (pH 4.5). Criteria for the establishment of diabetes were weight loss, polyuria, glucosuria, and hyperglycemia. Animals not meeting these criteria were not used in experiments. Twelve nondiabetic controls were sham-injected.

Three days after injection of streptozotocin, diabetic animals were divided into two weight-matched groups: untreated diabetic and insulin-treated diabetic. There were 12 rats in each group. The insulin-treated diabetic rats received daily injections of NPH insulin (3–4 U, s.c.) for an additional 12 days.

One day before they were killed, urine was collected from 5–6 rats from each of the three groups over a 24-h period for measurements of glucose, creatinine, calcium (DuPont Automatic Clinical Analyzer, Wilmington, Delaware), phosphorus,[11] and cyclic AMP.[12] At the time of death (15th day after streptozotocin or sham injection), blood from all animals was obtained for the determination of serum glucose, calcium, phosphorus, and creatinine. Serum iPTH was measured using a nonequilibrium RIA procedure modified from Conaway and Anast.[13] The standard curve used in these experiments was made by dilution of pooled sera from vitamin-D-deficient rats; these sera contain a high level of iPTH. Multiple dilutions of individual samples gave standard curves parallel to that of the standard sera. The lower limit of detectability of this assay was 1.4 μl eq/ml serum. The antiserum used was chicken antibovine PTH (CH 977).[14] Each sample was assayed in duplicate 100-μl aliquots. A third 100-μl aliquot was assayed in the absence of antiserum to correct for nonspecific binding. Bound and free hormone were separated with dextran-coated charcoal. Serum iCT was measured by RIA[15] using goat antibody to human CT and human CT as standards. The lower limit of detectability of this assay was 100 pg/ml.

The renal production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 was measured in vitro using isolated renal cortical slices as previously described.[9] Briefly, kidneys were removed from rats, bisected in the sagittal plane, and cortical slices (50–75 mg/slice) were prepared from the kidney with a Stadie-Riggs microtome. Slices were weighed and placed in plastic vials (2 slices per vial) containing 1 ml Krebs-Ringer bicarbonate buffer (pH 7.4). The vials were gassed with 95% O_2/5% CO_2 for 1 min and stoppered. The buffer contained unlabeled 25-OH-D_3 and tritium-labeled 25-OH-D_3 (25-OH-[26,27-^{3}H]-D_3) purchased from Amersham Corp. (Arlington Heights, Illinois). The concentration of unlabeled 25-OH-D_3 (a gift of Dr. J. C. Babcock, The Upjohn Co., Kalamazoo, Michigan) was 0.25 μM. The buffer concentration of Ca was 2.5 mM and the P concentration was 1.2 mM. After 1 h of incubation at 37°C in a shaking water bath, slices were extracted and analyzed for tritiated vitamin D metabolites using HPLC. The tritiated 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 metabolites were routinely identified by co-migration with standard vitamin D compounds on a Zorbax-SIL column (0.6 × 25 cm) equilibrated with hexane/methanol/methylene chloride (8:1:1) (see Figure 2). These tritiated metabolites also comigrate with standard vitamin D compounds on a reverse phase μ Bondapak C18 column (0.6 × 25 cm) equilibrated with methanol/water (4:10), and the metabolites show the expected periodate sensitivity. Production of 25-OH-D_3 metabolites was expressed as pg per milligram slice weight per minute. Renal production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 has been shown to be linear with time (0–90 min) and tissue weight (40–250 mg) using this procedure.[9]

The response of the renal adenylate cyclase system to PTH was determined by measuring the increase in tissue content of cyclic AMP in the presence of PTH as previously described.[16] Three rats from a diabetic group and three rats from a nondiabetic control group were used in this experiment. Slices of renal tissue were prepared as described for the measurement of 25-OH-D_3 metabolism and preincubated for 25 min in 1.0 ml Krebs-Ringer bicarbonate buffer containing 1 mM 3-isobutyl-1-xanthine (MIX), a cyclic nucleotide phosphodiesterase inhibitor. PTH (5 U/ml) was then added to the slice incubation medium. After 5 min, renal slices were homogenized in 0.5 ml of 50 mM sodium acetate buffer, pH 4.0, and heated at 95°C for 10 min. The supernate of the homogenate was assayed for cyclic AMP using the protein-binding method of Gilman.[12] Tissue content of cAMP was expressed as pmol per milligram wet weight of tissue. Under these incubation conditions, tissue content of cyclic AMP reflects tissue adenylate cyclase activity, since MIX has been shown to completely inhibit cyclic nucleotide phosphodiesterase activity.[16]

Results are given as the mean value ± SEM. Student's two-tailed *t* test[17] was used for comparison of differences. A confidence level of 95% or greater was considered significant.

RESULTS

Initial body weights were similar (Table 1), but diabetic rats lost 16% of their body weight by 16 days after streptozotocin treatment. Control and insulin-treated diabetic animals gained 35% and 46% of body weight, respectively. Diabetic rats had marked hyperglycemia and glucosuria, which was corrected by insulin treatment. The mean serum calcium and phosphorus concentrations were not significantly different among groups.

TABLE 1
Body weight, serum glucose, calcium, phosphorus, iPTH, iCT, urinary volume, and glucose

	Control	Diabetic	Insulin-treated
Body weight (g)			
Initial	128 ± 2	126 ± 2	125 ± 3
Final	172 ± 3	106 ± 5*	183 ± 5†
Serum glucose (mg/dl)	142 ± 4	563 ± 38*	89 ± 17†
Ca (mg/dl)	10.8 ± 0.1	9.6 ± 0.6	10.5 ± 0.7
P (mg/dl)	10.3 ± 0.4	11.9 ± 0.9	11.4 ± 2.1
iPTH (μl/eq/ml)	134 ± 22	172 ± 61	196 ± 30
iCT (pg/ml)	322 ± 57	84 ± 37*	679 ± 110*,†
Urine			
Volume (ml)	15 ± 2	129 ± 12*	21 ± 3†
Glucose (mg/dl)	6 ± 1	1253 ± 68*	7 ± 2†

Table entries are the mean ± SEM of 7–12 rats per group.
*Significantly different from control ($P < 0.05$).
†Significantly different from diabetic ($P < 0.05$).

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Serum iPTH levels of each group were not significantly different from each other (Table 1). The high levels of serum iPTH reflected a response of the parathyroid glands to the low calcium diet. Serum iCT levels were significantly lower in diabetic rats compared with controls. Insulin-treated diabetics have iCT values significantly greater than those of diabetic or control rats.

The conversion of $[^3H]$25-OH-D_3 to $[^3H]$1,25-$(OH)_2$-D_3 and $[^3H]$24,25-OH_2-D_3 by renal slices was studied in control, diabetic, and insulin-treated diabetic rats. The results are shown in Figure 1. Diabetic rats showed a marked drop in 1,25-$(OH)_2$-D_3 production and a rise in 24,25-$(OH)_2$-D_3 production compared with controls. With insulin replacement therapy, the diabetic rats showed a significant increase in 1,25-$(OH)_2$-D_3 production and decrease in 24,25-$(OH)_2$-D_3 production compared with untreated diabetic rats. Representative HPLC profiles of extracts of renal slices are shown in Figure 2. After a 1-h incubation with tritiated 25-OH-D_3, slices from a diabetic rat contained only small amounts of tritiated 1,25-$(OH)_2$-D_3 and large amounts of tritiated 24,25-$(OH)_2$-D_3. In contrast, slices from an insulin-treated diabetic rat contained a significantly larger amount of 1,25-$(OH)_2$-D_3 and a much smaller amount of 24,25-$(OH)_2$-D_3.

The effect of diabetes and insulin treatment on renal function was examined. There was no significant difference in creatinine clearance among the groups (Table 2). Diabetic rats excreted a markedly increased amount of urinary calcium and phosphorus compared with the controls. Insulin-treated diabetic rats demonstrated no hypercalciuria or hyperphosphaturia. Control rats and insulin-treated diabetic rats excreted similar amounts of urinary cyclic AMP. Urinary cyclic AMP excreted by diabetic rats was significantly greater than those of control or insulin-treated diabetic animals indicating that urinary cyclic AMP was not decreased in diabetic rats. Further examination of renal cyclic AMP responsiveness to PTH was carried out in vitro. We found no difference in cyclic AMP response to PTH by renal slices from diabetic rats compared to the response of control tissues (Table 3). PTH had a similar stimulatory effect in slices from both groups of animals.

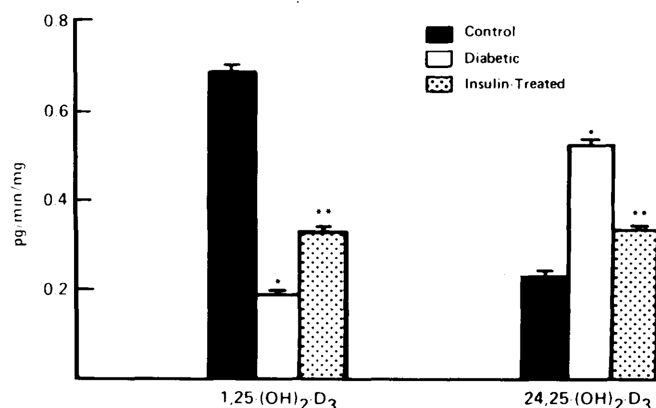

FIGURE 1. Renal slice production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 by control (solid bar), diabetic (open bar), and insulin-treated diabetic (stippled bar) rats (4–7 rats in each group). *Significantly different from control ($P < 0.05$). **Significantly different from diabetic ($P < 0.05$).

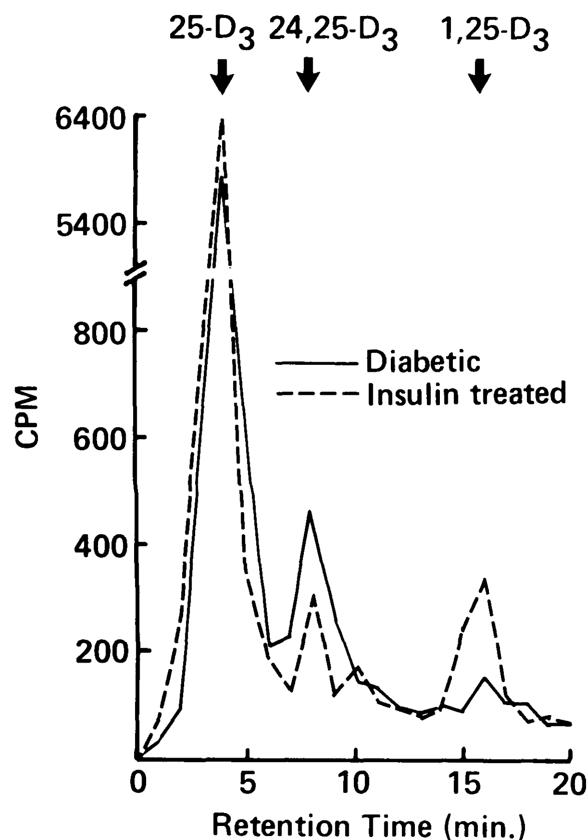

FIGURE 2. HPLC profiles of extracts of renal slices from diabetic and insulin-treated diabetic rats. Renal slices from diabetic and insulin-treated diabetic rats were incubated with tritiated 25-OH-D_3 for 1 h and extracted. Extracts, containing 10,000 cpm each, were chromatographed using a Zorbax-SIL column (0.6 × 25 cm) equilibrated with hexane/methanol/methylene chloride (8:1:1) at a flow rate of 1.1 ml/min. 1.1-ml fractions were collected and analyzed for radioactivity by scintillation counting. The position of the 254 nm absorbance of standard vitamin D compounds is indicated at the top of the figure.

DISCUSSION

The results of our studies demonstrate that the synthesis of 1,25-$(OH)_2$-D_3 by the kidney is severely impaired in streptozotocin-induced diabetes in the rat (Figure 1). This impairment did not correlate with alterations in serum calcium, phosphorus or parathyroid hormone, since there was no difference in these parameters among the groups studied (Table 1).

Diabetic rats had significant calciuresis and phosphaturia (Table 2). This has been attributed to osmotic diuresis.[18,19] Insulin therapy returned calcium and phosphorus excretion to normal (Table 2). Total urinary cyclic AMP excretion by the diabetic group was increased in our studies (Table 2). However, nephrogenous cyclic AMP was not determined. The total urinary cyclic AMP excretion by diabetic rats was significantly higher than the excretion by the other two groups. The elevated urinary cyclic AMP may be due to the elevated levels of circulating glucagon and catecholamines usually found in poorly controlled diabetics.[20,21] Another possible cause of this increased urinary cyclic AMP excretion may be secondary hyperparathyroidism due to renal calcium loss. Serum iPTH levels from diabetic rats were higher than those from the controls. However, the difference was not significant.

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TABLE 2
Creatinine clearance, urinary calcium, phosphorus, and cyclic AMP

	Control	Diabetic	Insulin-treated
Creatinine clearance (ml/min)	1.08 ± 0.12	0.76 ± 0.11	1.14 ± 0.12
Urinary			
Ca (mg/24 h)	0.68 ± 0.03	2.76 ± 0.07*	0.82 ± 0.03†
P (mg/24 h)	13 ± 2	106 ± 10*	17 ± 3†
cyclic AMP (nmol/100 ml GF)	13.1 ± 1.6	21 ± 3.4‡	10.7 ± 0.1§

Table entries are the mean ± SEM of 5–6 rats per group.
*Significantly different from control ($P < 0.001$).
†Significantly different from diabetic ($P < 0.001$).
‡Significantly different from control ($P < 0.025$).
§Significantly different from diabetic ($P < 0.025$).

Similar changes in urinary calcium and urinary cyclic AMP excretion in diabetes mellitus have been noted in humans.[22]

Renal resistance to parathyroid hormone in the streptozotocin-induced diabetic rat has been suggested as the mechanism by which the 1α-hydroxylase is depressed.[10] This was based on the findings that the streptozotocin-induced diabetic rats excreted significantly less nephrogenous cyclic AMP compared with the controls or the insulin-treated diabetics.[10] We evaluated the renal responsiveness to PTH by measuring cyclic AMP accumulation by the renal slices following addition of PTH directly to the kidney slice. We found that the kidneys of streptozotocin-induced diabetic rats elicited a normal cyclic AMP response to PTH (Table 3).

Our investigation shows that insulin therapy markedly improves the impaired renal 1,25-$(OH)_2$-D_3 synthesis in diabetic rats (Figures 1 and 2). The data do not establish, however, that insulin acts directly on the renal 1α-hydroxylase. Insulin apparently has permissive effect on PTH stimulation of 1,25-$(OH)_2$-D_3 production in cultured kidney cells.[23] This finding suggests that insulin deficiency in diabetic state is responsible for the decreased 1,25-$(OH)_2$-D_3 production. However, insulin is probably not an absolute requirement in 1α-hydroxylation.[21] Diabetic rats when fed a low (0.02%) calcium diet can significantly increase serum 1,25-$(OH)_2$-D_3 concentration compared with diabetic rats fed a normal (0.5%) calcium diet.[24]

Serum iCT levels were elevated in insulin-treated diabetic rats in our study as well as that of others,[25] and serum iCT was reduced in diabetic rats relative to controls. Calcitonin has been shown to selectively stimulate the 1α-hydroxylase in proximal straight tubule of rat kidney by a mechanism independent of adenylate cyclase activation.[26] It is tempting to postulate that the effect of insulin on the renal 1α-hydroxylation may be mediated, in part, by increased levels of circulating calcitonin. However, the evidence for a direct role of calcitonin on vitamin D metabolism is still lacking.[27]

TABLE 3
Cyclic AMP production by renal slices from diabetic and nondiabetic control rats

	cyclic AMP (pmol/g wet weight)	
Condition	Diabetic	Control
Without PTH	4.1 ± 0.6	4.7 ± 0.3
With PTH	39.8 ± 3.3*	39.7 ± 4.6*

Table entries are the mean ± SEM of 6 slices from 3 rats per group.
*Significantly different from "without PTH" ($P < 0.01$).

The possibility of streptozotocin nephrotoxicity as the cause of depressed 1,25-$(OH)_2$-D_3 production in the diabetic rats is not likely for several reasons. First, renal slices from diabetic rats are capable of increasing production of 24,25-$(OH)_2$-D_3, indicating the integrity of the mitochondria, which are the proposed site of 1,25-$(OH)_2$-D_3 synthesis. Second, with insulin therapy, renal slices from diabetic rats showed an increase in the level of 1,25-$(OH)_2$-D_3 production. Third, diabetic rat kidneys showed normal responsiveness to PTH in terms of cyclic AMP generation. Fourth, pretreatment with nicotinamide prevents diabetogenic effect of streptozotocin. Renal slices from rats treated with nicotinamide prior to streptozotocin injection produced 1,25-$(OH)_2$-D_3 in the nondiabetic control ranges (unpublished data).

In conclusion, this study clearly demonstrates that streptozotocin-induced diabetes in the rat is associated with decreased renal production of 1,25-$(OH)_2$-D_3. Insulin deficiency is likely to be responsible, at least in part, for the reduced production of this calcitropic hormone. The defect or defects lie beyond the renal generation of cyclic AMP. The mechanism by which insulin restores the renal 1α-hydroxylase activity remains unknown.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Cindy Gross and Britt Thomas and the secretarial assistance of Sharon Smith.

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