Bone and Mineral Metabolism in BB Rats With Long-Term Diabetes

Decreased Bone Turnover and Osteoporosis

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The effect of long-term diabetes mellitus on bone and mineral metabolism was studied in BB rats. Diabetic rats were treated with 1 U of long-acting insulin every other day for 12 wk and compared with nondiabetic littermates. Urinary calcium excretion was increased >10-fold, but serum total and diffusible calcium remained normal. Serum concentrations of both 1α,25-dihydroxyvitamin D_3 and vitamin D–binding protein were significantly decreased in diabetic rats. The intestinal calbindin-D 9K concentration was decreased by nearly 50%, and active duodenal calcium absorption was totally abolished. Trabecular bone volume measured in the tibial metaphysis was decreased by 44%, and the osteoblast and osteoid surfaces were <10% of values observed in control rats, whereas the osteoclast surface was unchanged by diabetes. The daily bone formation (bone mineral apposition rate) measured by labeling twice with calcein was decreased by 86% in diabetic rats. The serum concentration of osteocalcin, a biochemical marker of osteoblast function, was similarly decreased (mean ± SE 23 ± 3 and 62 ± 4 μg/L in diabetic [*n* = 15] and nondiabetic [*n* = 15] rats, respectively). Serum osteocalcin was significantly correlated with the serum concentration of insulinlike growth factor I (*r* = 0.89, *P* < 0.001). Bone strength measured as the energy needed to fracture the femur was markedly decreased (5.3 ± 1.4 and 8.4 ± 1.3 N · m · degree in diabetic and nondiabetic rats, respectively; *P* < 0.01). These histological, chemical, and biomechanical data clearly indicate that long-standing diabetes in BB rats results in severe low-turnover osteoporosis probably related to decreased osteoblast recruitment and/or function. ***Diabetes*** **39:477–82, 1990**

Insulin-dependent diabetes mellitus (IDDM) in humans is frequently associated with osteoporosis (1–3), although an increased frequency of fractures has not been observed in a mixed group of IDDM and non-insulin-dependent diabetic subjects (4).

Studies in young streptozocin-induced diabetic (STZ-D) rats have more unanimously demonstrated decreased bone growth and strength (5–8). However, the pathogenetic mechanisms are either largely unknown or disputed and vary among increased urinary calcium loss (9), decreased intestinal calcium absorption (10), abnormal vitamin D metabolism (11), abnormal collagen metabolism (12), microangiopathy (13), and primary dysfunction of the osteoblast (7, 14–18). Most previous studies have dealt with short-term diabetes induced by a potentially nephrotoxic drug (STZ). Therefore, we used spontaneously diabetic BB rats to investigate the effects of diabetes on bone and mineral metabolism. The use of this animal model has many advantages: it avoids the use of toxic diabetogenic drugs, and the nature and prepubertal onset of the disease largely correspond to what is observed in human IDDM.

After 3–4 wk of untreated diabetes, we found a slight decrease in tibial bone mass but normal trabecular bone volume in the tibial metaphysis and normal strength of the femur. The serum concentration of osteocalcin, a marker of bone formation, was markedly reduced (20% of control rats) and correlated with decreased osteoblast number and bone mineral apposition rate by dynamic histomorphometry; the osteoclast surface was also reduced but less than the osteoblast surface (18).

We hypothesized that, if the osteoblast number and/or function remained more affected than the osteoclast number, decreased bone volume and reduced bone strength would develop if diabetes duration was sufficiently long. In this study, we found continuously decreased bone formation and marked osteopenia in poorly treated diabetic BB rats after 12 wk of diabetes.

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Received for publication 16 August 1989 and accepted in revised form 29 November 1989.

RESEARCH DESIGN AND METHODS

Male BB rats were handled as described previously (18–20) and had free access to food and water. Animals were fed a standard laboratory diet containing 0.9% calcium, 0.62% phosphate, and 2000 IU vitamin D/kg (Hope Farms, Woerden, The Netherlands). Body weight and urinary glucose and ketones were frequently measured in the diabetic rats to exclude "stable" diabetic rats (21). To avoid frank ketoacidosis, however, they were given 1 U of long-acting insulin (monocomponent ultralente, Novo, Bagsvaerd, Denmark) three times a week. All studies were performed after a diabetes duration of 12 wk.

In one group of rats, blood samples were taken from the abdominal aorta after pentobarbital sodium anesthesia (60 mg/kg i.p.). Duodenal mucosal scrapings and kidneys for calbindin D measurements were rapidly taken after blood sampling, snap frozen in liquid N_2, and stored at −80°C. The left tibia was taken for histomorphometry, the left femur was used for osteocalcin measurements, and the right tibia and femur and the four distal lumbar vertebrae were used for chemical analysis. In a second group of rats, active duodenal calcium absorption was evaluated by the everted gut sac technique (20). The right leg of these rats was disarticulated, snap frozen in liquid N_2, and stored at −80°C for studies of mechanical properties of bone.

Assays. Plasma glucose was measured by the glucose oxidase method (Beckman Glucose Analyzer 2, Fullerton, CA). Insulin was measured by radioimmunoassay (RIA) with a rat insulin standard. Insulinlike growth factor I (IGF-I) was determined by RIA in acid-ethanol–extracted plasma. Recombinant human IGF-I was kindly donated by Ciba-Geigy (Basel), and antibodies were raised in a guinea pig (inter- and intra-assay coefficients of variation of 7.7 and 7.4%, respectively).

Calcium was measured by atomic absorption spectroscopy, and total protein was measured by the biuret method. The diffusible calcium levels in plasma were then calculated according to Zeisler (22).

The serum concentrations of 25-hydroxyvitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), and vitamin D–binding protein (DBP) were determined by competitive protein-binding assay, RIA, and single radial immunodiffusion, respectively; the free–1,25-$(OH)_2D_3$ index was calculated as the 1,25-$(OH)_2D_3$–DBP molar ratio (23). Osteocalcin was measured by RIA in plasma and bone (24). Calbindin-D 9K concentration was measured by RIA in the duodenal mucosal scrapings, calbindin-D 28K was measured in the kidneys with calbindin-D 9K purified from rat duodenum, and calbindin-D 28K was measured from rat kidneys as reference standards (25,26).

Bone histomorphometry. An intraperitoneal injection of calcein (1 g calcein and 2 g $NaHCO_3$ in 100 ml water; 0.16 g/kg) was given 11 days and 1 day before death. The processing of the tibiae and methods for histomorphometrical analysis have been described in detail (18).

The following parameters were measured: epiphyseal width; bone volume, the percentage of trabecular bone (mineralized and not mineralized) expressed as a fraction of a volume unit of integral trabecular bone (bone and bone marrow); osteoid surface, the percentage of the circumference of trabecular bone covered with osteoid; osteoblast surface, the percentage of the circumference line of trabecular bone covered with cubic osteoblasts; and osteoclast surface, the percentage of the circumference line of trabecular bone covered with osteoclasts. For histodynamic measurements, the distance between the two calcein lines was measured in unstained sagittal sections (4 μm thick), 6 mm from the proximal end of the tibia, over the next 3-mm area. At each endosteal side, 10 measurements were made with fluorescent light and the image-analyzing computer. The daily mineral apposition rate was calculated from these data. If only one line was present, the distance was indicated as zero.

Bone chemical analysis. The right femur and tibia and the four distal lumbar vertebrae were used for chemical analysis. The adhering tissue of the vertebrae was removed by warming the vertebrae in a papain solution, whereas the tibia and femur were dissected free of adhering tissue. Fat-free bones were obtained by extraction in pure ethanol and diethyl ether. The bones were dried for 24 h at 100°C to obtain a dry bone weight and then were washed for 24 h at 600°C in a muffle furnace. The ashed bones were weighed and dissolved in 12 M HCl; calcium was determined in ash dilutions and was expressed as content in total bone and concentration per dry bone weight.

Bone mechanical properties. The femur was slowly thawed, potted in cerrobend alloy (Jackson Walter, King of Prussia, PA), mounted in the grips of a rapid-load torsion tester, and loaded to failure. Torsional strength, angular deformation, energy storage capacity, and stiffness were evaluated; in addition, changes in length and diaphyseal diameter were determined with a micrometer (reported values represent the average of anteroposterior and mediolateral dimensions). Data were recorded on an oscilloscope and photographed and quantified on an IBM personal computer (8,18).

Statistical analysis. All data are expressed as means ± SE. A computer program (Minitab, Statistics Dept., Pennsylvania State Univ., State College) was used. Intergroup differences were analyzed by one-way analysis of variance, and when significant ($P < 0.05$), the Welch test was used to compare pairs of means.

RESULTS

After 12 wk of diabetes, the adult diabetic BB rats had lost considerable weight; their final weight was only 60% of that of control rats (Table 1). Diabetic rats were severely hyperglycemic and had high food and water intakes and high urinary output. Their insulin levels were low but detectable, due to the low-dose insulin treatment; their circulating IGF-I concentrations were markedly decreased.

Calcium and vitamin D metabolism. Despite hypercalciuria, diabetic rats maintained normal total and diffusible plasma calcium levels (Table 2). Although 25-hydroxyvitamin D_3 concentrations were normal in diabetic rats, their 1,25-$(OH)_2D_3$ levels were only 60% of control levels; however, the serum concentration of the DBP was accordingly decreased, so that the free–1,25-$(OH)_2D_3$ index was not different from that in control rats (Table 2).

The duodenal mucosal calbindin-D 9K concentrations of diabetic rats were 52% of the concentrations in nondiabetic rats and corresponded to a similar decrease in the active calcium absorption in the duodenum, as evaluated by the

TABLE 1
General data on BB rats

	Control		Diabetic	
	Mean ± SE	*n*	Mean ± SE	*n*
Final weight (g)	450 ± 10	15	280 ± 10	15
Weight gain/loss over 12 wk (g)	83 ± 14	15	−61 ± 11	15
Food intake (g/day)	15 ± 1	10	33 ± 2	10
Urinary volume (ml/day)	5.9 ± 0.5	15	78 ± 13	17
Plasma glucose (mM)	10.3 ± 0.4	15	29.7 ± 1.2	16
Plasma insulin (pM)	1149 ± 158	13	100 ± 14	16
Plasma IGF-I (μg/L)	585 ± 22	10	190 ± 22	10

n, Number of observations. IGF-I, insulinlike growth factor I. $P < 0.001$ vs. control rats for each value.

everted gut sac technique. However, the renal calbindin-D 28K concentrations were significantly higher in diabetic than in control rats (Table 2).

Bone histomorphometry. The tibial epiphyseal width was decreased in diabetic rats (Table 3). The trabecular bone volume density, measured at the tibial metaphysis, was even more decreased (56% that of control rats). By static histomorphometry, the osteoblast and osteoid surfaces were markedly reduced (~10% that of control rats), whereas the osteoclast surface was normal in diabetic rats. Dynamic morphometry confirmed the low bone mineral apposition rate in diabetic rats (13% that of control rats).

Bone chemical analysis. The femur, tibia, and the four distal lumbar vertebrae of diabetic rats had lower (wet and dry) weights and lower calcium contents than nondiabetic rats (Table 4). However, no difference could be found in the relative ash and calcium contents of bones of rats with long-term diabetes.

Osteocalcin studies. Plasma osteocalcin concentrations in diabetic rats were only 37% of values found in nondiabetic rats, and circulating osteocalcin levels correlated with IGF-I concentrations ($r = 0.89$, $P < 0.001$; Fig. 1). The total osteocalcin content of the whole femur was reduced in diabetic rats, but the relative osteocalcin concentration (per dry wt) of the femur was not significantly different from that of control rats (Table 5).

Bone mechanical properties. The length and diaphyseal diameter of the femurs were slightly but not significantly decreased in diabetic rats (Table 6). However, all mechanical parameters (i.e., angular deformation, stiffness, torsional strength, energy absorption) were significantly decreased in the femurs of rats with long-term diabetes.

DISCUSSION

This study demonstrates that long-term diabetes (3 mo) in BB rats is accompanied by significant osteoporosis demonstrated by histology, chemical analysis, and biomechanical measurements of bone. Indeed, the total trabecular bone volume measured at the tibial metaphysis was only 56% that of the control rats (Table 3). The total calcium content of bones with largely cortical bone (tibia and femur) was decreased by 22 and 17%, whereas the calcium content of four lumbar vertebrae was decreased by 24% (Table 4). Moreover, bone strength, stiffness, energy storage capacity, and the ability to deform under loading were decreased in BB rats with long-term diabetes (Table 6). Although we did not measure osteoid volume directly, the unchanged relative mineral concentrations in the different bones (Table 4), the normal femoral osteocalcin concentration (Table 5), and the decreased osteoid surface (Table 3) exclude osteomalacia. The diabetic bone abnormality should therefore be classified as a form of secondary osteoporosis. Osteomalacia had first been suspected when disturbances in vitamin D metabolism and action were observed in STZ-D rats, i.e., decreased duodenal calcium absorption (10), decreased duodenal mucosal calbindin D concentrations (27), and decreased serum $1,25\text{-}(OH)_2D_3$ concentrations (7,11,19,28,29). We confirmed these changes in vitamin D metabolism in short-term diabetes in STZ-D rats (29) and BB rats with short-term (20) and long-term diabetes (Table 2); however, we believe that these changes in vitamin D concentrations are secondary to a decrease in the serum concentration of the DBP (20,29) and the large food (and thus calcium) intake of diabetic rats, resulting in high passive calcium absorption (20) and normal diffusible calcium concentrations in the plasma of diabetic

TABLE 2
Calcium and vitamin D metabolism in BB rats

	Control		Diabetic	
	Mean ± SE	*n*	Mean ± SE	*n*
Plasma calcium (mg/dl)				
Total	10.6 ± 0.1	14	10.2 ± 0.3	14
Diffusible	5.2 ± 0.2		5.6 ± 0.6	
Urinary calcium (mg/day)	0.50 ± 0.04	12	5.6 ± 0.5*	11
25-Hydroxyvitamin D_3 (μg/L)	9.3 ± 1.0	15	11.9 ± 0.8	14
1α,25-Dihydroxyvitamin D_3 (ng/L)	137 ± 15	11	87 ± 9†	10
Vitamin D–binding protein (mg/L)	550 ± 18	14	323 ± 20*	12
Free–1α,25-dihydroxyvitamin D_3 index‡	3.1 ± 0.3	11	3.9 ± 0.7	10
Duodenal mucosal calbindin-D 9K (μg/mg protein)	5.7 ± 0.9	7	3.0 ± 0.5†	7
Renal calbindin-D 28K (μg/mg protein)	3.9 ± 0.7	12	5.6 ± 0.5*	12
Duodenal calcium transport (serosal-mucosal ratio)	1.57 ± 0.10	11	0.85 ± 0.07*	12

n, Number of observations.
*$P < 0.001$, †$P < 0.05$, vs. control rats.
‡Molar ratio of 1α,25-dihydroxyvitamin D and vitamin D–binding protein multiplied by 10^5.

TABLE 3
Histomorphometrical data from tibial metaphysis in BB rats

	Control (n = 16)	Diabetic (n = 18)
Epiphyseal width (μm)	6.3 ± 0.2	5.3 ± 0.4*
Bone volume (%)	12.6 ± 0.7	7.1 ± 0.9†
Osteoid surface (%)	3.3 ± 0.9	0.2 ± 0.2‡
Osteoblast surface (%)	2.7 ± 0.7	0.2 ± 0.2‡
Osteoclast surface (%)	0.7 ± 0.1	0.9 ± 0.5
Bone mineral appositional rate (μm/day)	2.2 ± 0.3	0.3 ± 0.1†

Values are means ± SE. For definition of histomorphometrical analysis measurements, see RESEARCH DESIGN AND METHODS.
*$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$, vs. control rats.

rats (Table 2). Direct measurements of ionized calcium have confirmed the calculations of diffusible calcium in diabetic BB rats (data not shown).

The divergent changes in duodenal calbindin-D 9K and renal calbindin-D 28K concentrations in diabetic rats may well be due to tissue-specific responses to humoral stimuli. Indeed, Tohmon et al. (30) found decreased synthesis of calbindin-D 28K in the duodenum of glucocorticoid-treated chicks, whereas its synthesis was increased in the kidney. However, the physiological significance of changes in renal calbindin-D 28K remains unclear.

Diabetic osteoporosis can best be explained by severely reduced bone formation without comparable reduction in bone resorption. Previous studies in chemically or virally induced diabetes in rats or mice indicated that short-term diabetes was associated with decreased osteoblast function evaluated by ^{45}Ca incorporation (31,32), dynamic bone histology (7,15,18,33), or osteocalcin measurements (16–18).

Although histological or biochemical signs of decreased osteoblast number and/or function can be detected within a few weeks after development of diabetes (17,18), a real deficit in total bone volume or calcium content requires a longer duration of diabetes, as in this study (Tables 3 and 4). Previous studies could not reveal such bone calcium deficit after 1 mo (18), whereas after 7 wk of diabetes, either

TABLE 4
Chemical analysis of bone in BB rats

	Control		Diabetic	
	Mean ± SE	n	Mean ± SE	n
Femur				
Wet weight (mg)	1133 ± 29	13	938 ± 18*	14
Calcium (mg)	162 ± 4	8	136 ± 5*	11
Calcium dry weight (%)	22.2 ± 0.3		22.5 ± 0.5	
Tibia				
Wet weight (mg)	711 ± 22	13	574 ± 13*	14
Calcium (mg)	104 ± 5	10	81 ± 2*	12
Calcium dry weight (%)	22.3 ± 0.3		22.0 ± 0.3	
Four lumbar vertebrae				
Wet weight (mg)	782 ± 26	17	614 ± 15*	22
Calcium (mg)	174 ± 5	16	133 ± 5*	22
Calcium dry weight (%)	21.9 ± 0.4		21.6 ± 0.5	

n, Number of observations.
*$P < 0.001$ vs. control rats.

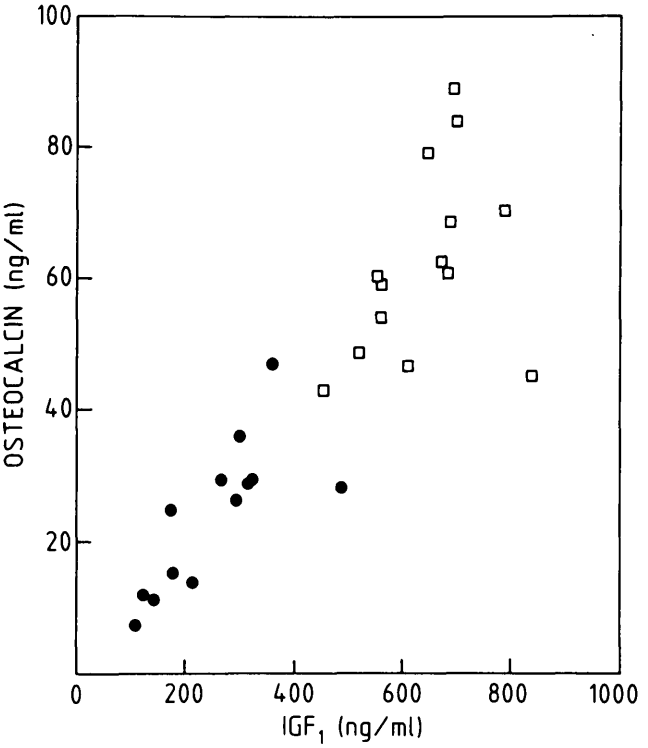

FIG. 1. Correlation between serum concentration of osteocalcin and insulinlike growth factor I (IGF_1) in diabetic (●) and nondiabetic (□) BB rats. $r = 0.89$, $P < 0.001$.

no decrease in calcium content (32) or a decrease in bone volume (17) was observed.

The arguments for decreased osteoblast number and/or function are numerous, e.g., low osteoblast and osteoid surfaces by bone histology of the tibial metaphysis (Table 3) and lumbar vertebrae (data not shown), low bone mineral apposition rate by dynamic bone histology, and low concentrations of the osteoblastic protein osteocalcin (Table 5). Moreover, 1,25-$(OH)_2D_3$ injections did not increase the osteocalcin levels in diabetic rats as they do in healthy rats, suggesting a real osteoblast dysfunction in diabetic animals (18). Because the osteoclast number either decreased slightly (after 1 mo of diabetes in BB rats; 18) or remained unchanged in rats with long-term diabetes (Table 3), osteoporosis must be the end point. The decreased calcium content of the bones also resulted in mechanically inferior bone, demonstrated by several biomechanical parameters, all in-

TABLE 5
Osteocalcin data in BB rats

	Control		Diabetic	
	Mean ± SE	n	Mean ± SE	n
Serum osteocalcin (μg/L)	62 ± 4	15	23 ± 3*	15
Femur				
Osteocalcin content (ng/femur)	622 ± 48	20	437 ± 28†	23
Osteocalcin concentration (ng/mg femoral dry wt)	0.84 ± 0.08	20	0.72 ± 0.04	23

n, Number of observations.
*$P < 0.001$, †$P < 0.05$, vs. control rats.

TABLE 6
Biomechanical properties of femurs in BB rats

	Control	Diabetic
Length (mm)	13.2 ± 2.9	12.6 ± 2.2
Diaphyseal diameter* (mm)	4.1 ± 0.3	3.9 ± 0.2
Angular deformation (degree)	14.8 ± 1.9	12.2 ± 2.7†
Stiffness (N · m · degree^{-1})	0.08 ± 0.02	0.06 ± 0.02†
Torsional strength (N · m)	0.97 ± 0.13	0.62 ± 0.16†
Energy absorption (N · m · degree)	8.4 ± 1.1	5.3 ± 1.4†

Values are means ± SE. n = 13 for each group.
*Mean of the maximal anteroposterior and lateral diameter of the midsection of the diaphysis.
†$P < 0.01$ vs. control rats.

dicating the brittleness of the diabetic bones after long-standing diabetes (Table 6), whereas after 1 mo of diabetes, no such abnormalities were yet detectable (18).

The effect of diabetes on the osteoblast number and/or function cannot be ascribed to malnutrition. Although undernourishment can reduce bone formation (18,34), we demonstrated that weight-matched nondiabetic rats did not show reduction in osteoblast number and osteocalcin concentration as did BB rats after 1 mo of diabetes (18). Other studies also demonstrated that the decreased collagen synthesis observed in diabetic rats can only partially (32%) be attributed to malnourishment (12). Low parathyroid hormone levels, such as found in 7-wk-old STZ-D rats (7), could also contribute to the low bone turnover.

The decreased concentration of IGF-I in the serum of diabetic rats was probably due to decreased synthesis because it is also found in several tissues of diabetic rats (unpublished observations). The urinary excretion of IGF-I was also increased in diabetic rats (122 ± 52 ng/day, n = 4) compared with nondiabetic rats (44 ± 9 ng/day), but these amounts cannot explain the observed differences in serum concentrations.

In view of the excellent correlation between the plasma concentrations of growth hormone–dependent and insulin-dependent IGF-I and several parameters of bone formation (bone mineral apposition rate and osteocalcin concentration) (Fig. 1), it is tempting to speculate that the deficiency of this growth factor in diabetes is at least partly responsible for decreased osteoblast recruitment and function (Table 1). IGF-I is a good potential pathogenetic intermediate, because osteoblasts contain receptors for IGF-I (35) and IGF-I can stimulate osteoblast replication and bone matrix synthesis (36,37). Articular collagen production was similarly found to be depressed in diabetic rats, and this depression was also highly correlated with serum IGF-I concentrations (12). On the other hand, insulin itself may also be important for bone cell function: insulin receptors are present in osteoblastic cells (38), and insulin was shown to promote the growth of an osteoblast like cell line (39). Intensive insulin treatment can totally correct (and even overcorrect) the biochemical and histodynamic bone abnormalities in diabetic BB rats (unpublished observations), and similar observations have been made after islet cell transplantation in STZ-D rats (40).

The finding that long-standing diabetes results in more-fragile bones may be relevant for clinical medicine. Although no increased incidence of skeletal fractures was found in a rather unselected American diabetic population (4), other studies from Sweden and Israel reported more fractures in diabetic subjects (13,41,42). More careful analysis of the incidence of osteoporosis and bone fractures in long-standing or postmenopausal IDDM is needed to evaluate the clinical implications of diabetic osteopenia. However, the diabetic animal model can be useful as a method to evaluate humoral, hormonal, or other pharmaceutical agents for their capability to stimulate bone formation in vivo.

In conclusion, diabetes mellitus results in decreased osteoblast number and bone mineral apposition. This osteoblast dysfunction remains evident even after 3 mo of poorly controlled diabetes and results in marked decreases in bone mineral content and bone strength. Diabetic osteoporosis is probably not due to primary abnormalities in calcium-regulating hormones but may be due to insulin deficiency or deficiency of insulin-dependent growth factors.

ACKNOWLEDGMENTS

This work was supported by the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (3.0029.85) and Novo Industri, Bagsvaerd, Denmark.

We thank Dr. W. Lissens for calcium measurements and R. van Bree, I. Jans, and N. Eb for skillful technical assistance.

This study was presented in abstract form at the 71st annual meeting of the Endocrine Society, Seattle, Washington, June 1989.

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