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Influence of High Glucose on 1,25-Dihydroxyvitamin D_3-Induced Effect on Human Osteoblast-like MG-63 Cells

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ABSTRACT

Impaired bone formation due to defective osteoblast function, as reflected in a decreased serum osteocalcin (OC) concentration in the patients with diabetes, has been implicated in the development of diabetic osteopenia. The role of hyperglycemia in this decrease in serum OC concentration was investigated. 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), an active form of vitamin D_3, stimulated OC secretion from the human osteosarcoma cell line MG-63 in a dose-dependent manner. Exposure of the cells to high concentrations of glucose for 7 days significantly impaired $1,25(OH)_2D_3$-induced OC secretion as compared with that observed with cells maintained under normal glucose (5.5 mM) or high mannitol conditions. The inhibitory effect of glucose was in a dose-dependent manner up to 55 mM. High glucose (55 mM) also attenuated the $1,25(OH)_2D_3$-induced increase in OC mRNA abundance in MG-63 cells, suggesting that the inhibition of the $1,25(OH)_2D_3$-induced increase in OC secretion by exposure to a high concentration of glucose was, at least in part, mediated at the transcriptional level. High glucose significantly decreased the number of $1,25(OH)_2D_3$ receptors in MG-63 cells, without any change in the dissociation constant for $1,25(OH)_2D_3$; this effect was not mimicked by high mannitol, indicating specificity for glucose. These observations suggest that a high glucose concentration significantly impairs the ability of osteoblastic cells to synthesize OC in response to $1,25(OH)_2D_3$ by reducing $1,25(OH)_2D_3$ receptor number, and that impaired cell function caused by sustained exposure to high glucose contributes to the defect in bone formation observed in the patients with diabetic osteopenia. (J Bone Miner Res 1995;10:1050–1056)

INTRODUCTION

PATIENTS WITH DIABETES mellitus may exhibit various disorders of calcium (Ca) metabolism, such as an impairment of Ca absorption[1,2] and loss of Ca from bone,[3] which can eventually produce diabetic osteopenia.[4–8] The severity of osteopenia depends on the quality of diabetic control.[9,10] Secondary hyperparathyroidism resulting from an increased loss of Ca into urine induced by hyperglycemia or glycosuria has been proposed as a major cause of the loss of bone mineral in diabetic osteopenia.[11–13] However, bone formation is not sufficiently increased in individuals with diabetes to compensate for the increase in bone resorption.[14] Thus, impaired bone formation due to an osteoblast deficit was recently proposed as being important in the development of diabetic osteopenia.[15,16] This hypothesis is supported by data showing a decrease in the serum concentration of osteocalcin (OC) in diabetic patients[17,18]; OC is produced specifically by osteoblasts and is thus a clinically useful marker for osteoblast function.[19] It is not known whether the decrease in serum OC concentration in diabetes results from the loss of insulin stimulation of osteoblasts[14,20] or from defective cell function induced by long exposure to high glucose concentrations. To address this question, we investigated the effects of sustained exposure of osteoblast-like cells to a high glucose concentration on OC synthesis induced by 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), a key hormone in bone mineralization.[21]

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The study was performed with the human osteosarcoma cell line MG-63, an osteoblast-like cell model.[22]

MATERIALS AND METHODS

Materials

Cell culture media, antibiotics, and trypsin were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY). 1,25-dihydroxy[26,27-^{3}H]vitamin D_3, [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Dupont/New England Nuclear (Boston, MA). Nonradioactive $1,25(OH)_2D_3$ was a gift from Chugai Pharmaceutical Co. (Tokyo, Japan). Diisopropylfluorophosphate (DFP), forskolin, 8-bromo-cAMP, and Kemptide were obtained from Sigma (St. Louis, MO), and menadione vitamin K_3 was from Wako Pure Chemical (Osaka, Japan). Human OC complementary DNA (cDNA) was obtained from Chiron Co. (Emeryville, CA). cDNAs of human OC and rat glyceraldehyde-3-phosphate (GAPDH)[23] were labeled with [α-^{32}P]dCTP with the use of random hexadeoxynucleotide primers. An OC immunoradiometric assay kit and a cAMP radioimmunoassay kit were obtained from Mitsubishi Chemical Co. (Tokyo, Japan) and from Yamasa Shouyu (Chiba, Japan), respectively. Buffers used were as follows: phosphate-buffered saline (PBS), 15 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (pH 8.0), 137 mM NaCl, and 2.7 mM KCl; $TEDK_{300}$, 50 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 5 mM dithiothreitol, and 300 mM KCl.

Cell culture

MG-63 cells, which show an osteoblastic phenotype,[22] were obtained from the American Type Culture Collection. The cells were cultured in 150-cm^2 flasks (Falcon, Oxnard, CA) with α-minimal essential medium (α-MEM) supplemented with 10% heat-inactivated FBS, penicillin (50 IU/ml), and streptomycin (50 μg/ml), and containing either 5.5 mM glucose (normal glucose), 55 mM glucose (high glucose), or 5.5 mM glucose plus 49.5 mM mannitol (high mannitol; an isosmolar control for high glucose). The cells were plated at a density of 3.8×10^3 cells/cm^2 and cultured for 7 days, by which time they had attained confluence. Culture medium was replaced by medium of the same composition on day 4.

Preparation of $1,25(OH)_2D_3$ receptors from MG-63 cells

MG-63 cells were processed essentially as described previously.[24–26] All operations were performed at 0–4°C. The cells were washed three times with Ca^{2+}- and Mg^{2+}-free PBS, suspended in $TEDK_{300}$ buffer with 5 mM DFP and sonicated three times (20 s each time) with intervals of 60 s. The cell lysate was centrifuged at 105,000g for 60 min to yield the receptor preparation, which was then frozen in liquid nitrogen and stored at −80°C until use. Protein concentration was determined by the method of Bradford[27] with bovine serum albumin (BSA) as the standard.

Measurement of $1,25(OH)_2D_3$ receptor abundance in MG-63 cells

$1,25(OH)_2D_3$ receptor abundance was determined by saturation analysis as described previously.[24] Portions (90 μl) of the MG-63 cell extract were incubated with various concentrations (0.01–1.0 nM) of $1,25(OH)_2$[26,27-^{3}H]D_3. Nonspecific binding was measured in the presence of a 100-fold excess of nonradioactive $1,25(OH)_2D_3$. The final concentration of ethanol in the mixture was 10%. The mixture was incubated on ice for 16 h, after which bound $1,25(OH)_2$[26,27-^{3}H]D_3 was determined with the hydroxyapatite assay.[28] Scatchard analysis was performed to determine the dissociation constant (K_d) and the maximal number of binding sites (B_{max}).

Determination of OC concentration in culture media

The effect of high glucose on OC secretion from MG-63 cells was examined. Cells were cultured for 7 days in medium containing the indicated concentrations of glucose and/or mannitol, with a medium change 96 h after cell inoculation. Cells were then cultured in the corresponding medium but containing 2% charcoal-treated FBS, ascorbic acid (50 μg/ml), 10 nM menadione vitamin K_3, and various concentrations of $1,25(OH)_2D_3$. FBS was treated with charcoal as described[22] to decrease the $1,25(OH)_2D_3$ concentration by 99%.[29] After 48 h of culture, the medium was collected and stored at −30°C until assayed for OC. The OC concentration of culture medium was determined by immunoradiometric assay[30] with two types of monoclonal antibodies that recognize the COOH-terminal residues 30–49 and midregion residue 12–33 of human OC.

Isolation and hybridization of RNA

Total RNA was isolated from MG-63 cells by acid guanidinium thiocyanate-phenol chloroform extraction,[31] and 20 μg were subjected to electrophoresis on a 1% agarose gel containing formaldehyde. The separated RNA molecules were transferred to a nylon filter (Hybond N; Amersham International, Amersham, U.K.), which was then incubated at 37°C for 16 h in a solution containing 50% formamide, 3× standard saline citrate, 50 mM Tris-HCl (pH 7.5), 0.1% SDS, tRNA (20 μg/ml), boiled salmon sperm DNA (20 μg/ml), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1× Denhardt's solution before hybridization with a ^{32}P-labeled OC or GAPDH cDNA probe in the same solution at 37°C for 40 h.[32] The filter was subjected to autoradiography, and the density of each band was determined by a laser densitometer (model 2222 Ultroscan XL; Pharmacia LKB, Uppsala, Sweden). The amount of OC mRNA was standardized relative to the amount of GAPDH mRNA and expressed in arbitrary units.

Determination of intracellular cAMP concentration in MG-63 cells

Intracellular cAMP was assayed as described previously.[33] Briefly, after culture for 7 days in medium containing normal glucose, high glucose, or high mannitol, MG-63 cells

were washed twice with PBS containing 10 mM theophylline and homogenized in 0.1 N HCl. The cAMP concentration in the cellular extract was determined by radioimmunoassay.

Assay of cAMP-dependent protein kinase activity in MG-63 cells

The activity of cAMP-dependent protein kinase (PKA) was assayed essentially as described.[34,35] Briefly, after culture for 7 days in medium containing normal glucose, high glucose, or high mannitol, MG-63 cells were washed twice with PBS and homogenized in a solution containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM ethyleneglycol-bis (β-aminoethylether) N, N, N′, N′, -tetraacetic acid (EGTA), and 0.1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was then centrifuged at 105,000g for 60 min to yield the enzyme extract. The extract was stored at −80°C until use. The assay was performed in a total volume of 100 μl containing 20 μl of solubilized cell supernatant (16 mg/ml), 40 mM Tris-HCl (pH 7.5), 10 μM [γ-^{32}P]ATP (2000–3000 cpm/pmol), 12.5 mM $MgCl_2$, and 3 mM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), in the presence or absence of 10 μM cAMP. After incubation for 10 min at 30°C, the reaction was terminated by immediate transfer of the tubes to ice and spotting 40 μl of the mixture in duplicate onto P-81 phosphocellulose paper (Whatman, Clifton, NJ). The P-81 paper was immediately dropped into 30% acetic acid and washed with three changes of this solution before a final wash with acetone. After drying, the paper was placed in vials containing 4 ml of ACS-2 for scintillation counting. Hormonal activation of PKA was expressed as an activity ratio,[35] defined as the ratio of PKA activity measured in the absence of added cAMP to that in the presence of a saturating concentration (10 μM) of cAMP.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed by the Bonferonni test, one-way analysis of variance followed by Scheffe's F test or Student's *t*-test. A p value of <0.05 was regarded as statistically significant.

RESULTS

Effect of high glucose on 1,25(OH)$_2$D$_3$-induced OC secretion from MG-63 cells

To determine whether a high glucose concentration by itself could impair the osteoblastic cell function, we investigated the effect of sustained exposure of MG-63 cells to high concentrations of glucose on 1,25(OH)$_2$D$_3$-induced OC secretion. To avoid the influence of different cell densities on the sensitivity of the cells to 1,25(OH)$_2$D$_3$,[22] we performed experiments after culture of the cells for 7 days, by which time the cultures had attained confluence. The DNA content per culture dish (9.6 cm^2) after 7 days under normal glucose (n = 3), high glucose (n = 3), and high mannitol (n = 3) conditions was 81.2 ± 6.0, 83.5 ± 4.47, and 78.0 ± 7.14 μg, respectively; the differences among these values did not reach statistical significance. 1,25(OH)$_2$D$_3$ increased OC secretion in a concentration-dependent manner up to a concentration of 100 nM under normal glucose, high glucose, and high mannitol conditions (Fig. 1). However, the response to 1,25(OH)$_2$D$_3$ of cells cultured at a high concentration of 55 mM glucose was significantly decreased relative to that of cells cultured under normal glucose or high mannitol conditions. These data indicate that high glucose itself impairs the ability of osteoblast-like cells to synthesize OC.

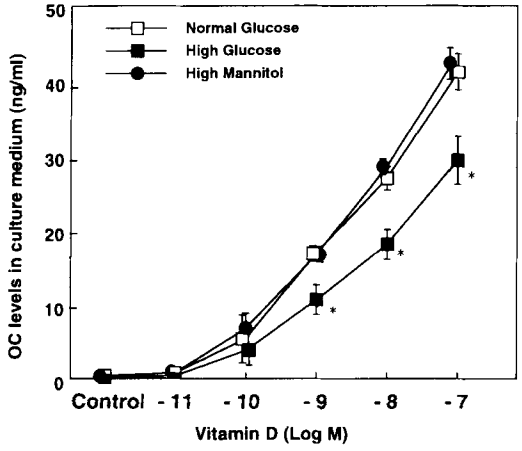

FIG. 1. Effect of high glucose on 1,25(OH)$_2$D$_3$-induced OC secretion from MG-63 cells. Cells were cultured for 7 days in medium containing normal glucose, high glucose, or high mannitol and then incubated in the absence (control) or presence of various concentrations of 1,25(OH)$_2$D$_3$ under the same conditions for another 48 h. The medium was collected and its content of OC determined. Data are means ± SD of quadruplicate determinations. *$p < 0.05$ versus corresponding concentration of 1,25(OH)$_2$D$_3$ under normal glucose or high mannitol (Bonferonni test).

To further support the specificity of the inhibitory effect of a high concentration of glucose on 1,25(OH)$_2$D$_3$-induced OC secretion, concentration-dependent effects were examined. As shown in Fig. 2, glucose significantly impaired OC secretion by 13.8 mM from the cells treated with 100 nM 1,25(OH)$_2$D$_3$ and the effect was in a concentration-dependent manner up to 55 mM. Therefore, in the further experiments, a glucose concentration of 55 mM was selected as high glucose condition, since 55 mM glucose seemed not to have a hyperosmolar effect as reflected by the lack of the effect of high mannitol, an isosmolar control of high glucose.

Effect of high glucose on OC mRNA abundance in MG-63 cells

The amount of OC mRNA was determined at various times after the addition of 100 nM 1,25(OH)$_2$D$_3$ to MG-63 cells that had been cultured under high glucose, or normal glucose conditions for 7 days. Northern blot analysis of total RNA with a human OC cDNA probe revealed a single, 0.6-kb band (Fig. 3), as previously described.[36] 1,25(OH)$_2$D$_3$ increased the amount of OC mRNA in a time-dependent

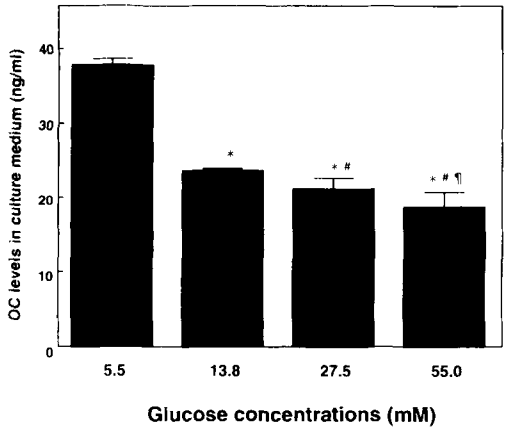

FIG. 2. Concentration-dependent effect of glucose on $1,25(OH)_2D_3$-induced OC secretion from MG-63 cells. Cells were cultured for 7 days in medium containing indicated concentrations of glucose and then incubated with 10^{-7} M $1,25(OH)_2D_3$ under the same conditions for another 48 h. The medium was collected for the determination of its content of OC. Data are means ± SD of five determinations and were statistically analyzed by analysis of variance followed by Scheffe's F test. $^*p < 0.05$ versus 5.5 mM glucose; $\#p < 0.05$ versus 13.8 mM glucose; $^¶p < 0.05$ versus 27.5 mM glucose.

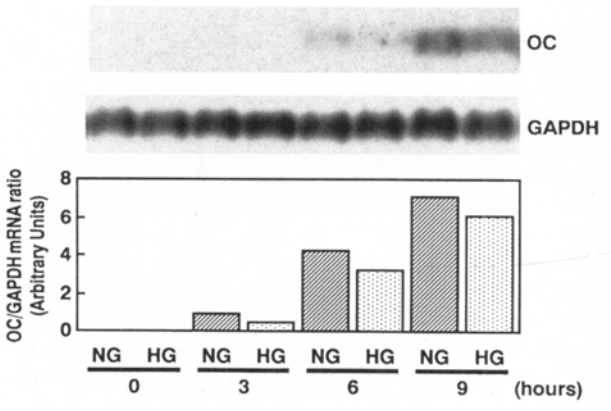

FIG. 3. Effect of high glucose on OC mRNA abundance in MG-63 cells. Cells were cultured for 7 days under normal (N) or high (H) glucose conditions and then exposed to 100 nM $1,25(OH)_2D_3$ under the same conditions for the indicated times. Total RNA was isolated and subjected to Northern blot analysis with ^{32}P-labeled human OC (upper panel) and rat GAPDH (middle panel) cDNA probes. Autoradiograms were scanned with a laser densitometer and the densities of OC mRNA bands were normalized relative to those of GAPDH mRNA bands (lower panel). Results are expressed in arbitrary units. Similar results were obtained in two separate experiments.

manner under both glucose conditions. However, when results were normalized to GAPDH mRNA abundance, high glucose attenuated the $1,25(OH)_2D_3$-induced increase in OC mRNA at 3, 6, and 9 h after the addition of $1,25(OH)_2D_3$. The data were reproducible in two separate experiments.

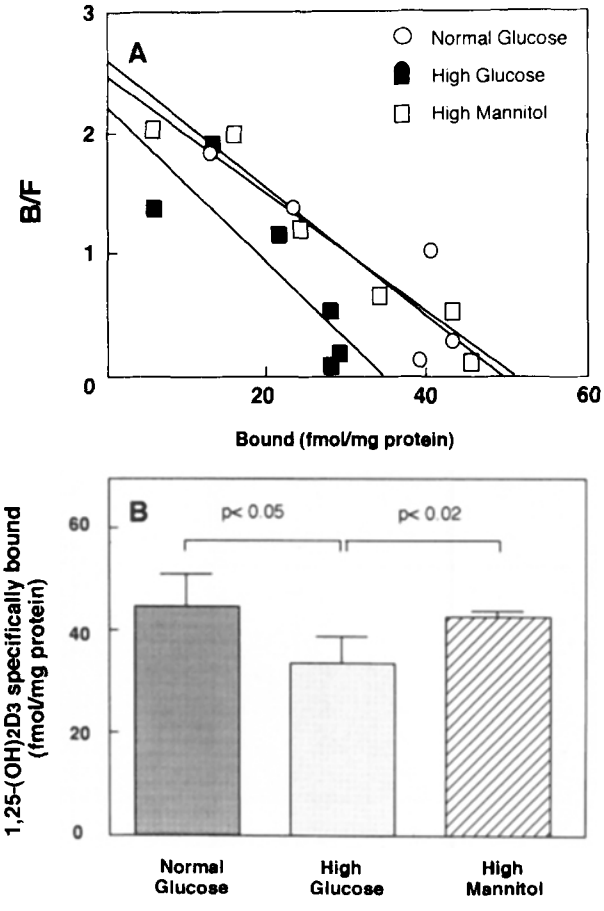

FIG. 4. Scatchard plot (A) and the B_{max} (B) of $1,25(OH)_2[26,27\text{-}^3H]D_3$ binding after treatment of MG-63 cells with normal glucose, high glucose, or high mannitol. Cells were cultured for 7 days under normal glucose, high glucose, or high mannitol conditions, and cell extracts were then prepared for measurement of $1,25(OH)_2D_3$ binding by a ligand binding assay. Data in (B) are means ± SD of five determinations in each group and were statistically analyzed by the Bonferonni test.

Effect of high glucose on $1,25(OH)_2D_3$ receptor number in MG-63 cells

$1,25(OH)_2D_3$ receptor abundance in MG-63 cells was determined with a ligand binding assay. MG-63 cells contained a single class of $1,25(OH)_2D_3$ receptors with characteristics similar to those of classical target tissues (Fig. 4A). High glucose decreased the B_{max} of $1,25(OH)_2[26,27\text{-}^3H]D_3$ binding sites by 17% (Fig. 4B) without any apparent change in K_d. High mannitol had no apparent effect on $1,25(OH)_2[26,27\text{-}^3H]D_3$ binding.

Role of cAMP and PKA in the regulation of $1,25(OH)_2D_3$ receptor number in MG-63 cells

We[37] and others[38] have shown that the cAMP-PKA system contributes to the regulation of $1,25(OH)_2D_3$ recep-

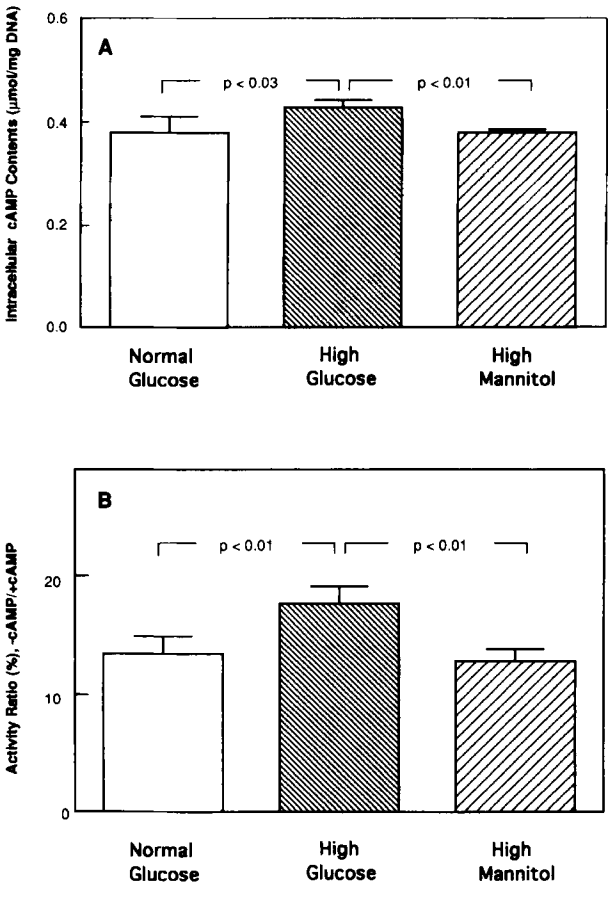

FIG. 5. Effect of high glucose on intracellular cAMP concentration (A) and the PKA activity ratio (B) in MG-63 cells. Cells were cultured for 7 days under normal glucose, high glucose, or high mannitol conditions, after which extracts were prepared and assayed for cAMP (upper panel) and PKA activity (lower panel). Data are means ± SD of four determinations. Statistical analysis was performed by Student's *t*-test.

tor number in human leukemic HL-60 cells and osteoblastic UMR-106 cells, respectively. Therefore, we investigated the effect of high glucose on intracellular cAMP concentration in MG-63 cells. The cAMP concentration was significantly higher in high glucose-treated cells than in cells cultured under normal glucose or high mannitol conditions (Fig. 5A). Consistent with the cAMP data, the PKA activity ratio was also significantly increased in cells treated with high glucose (Fig. 5B). However, dibutyryl cAMP did not affect $1,25(OH)_2D_3$ receptor number in MG-63 cells under either high or normal glucose conditions (data not shown). Similarly, neither forskolin nor 8-bromo-cAMP affected $1,25(OH)_2D_3$ receptor number (data not shown).

DISCUSSION

We have shown that exposure to high concentrations of glucose treatment significantly impaired the ability of osteoblast-like MG-63 cells to synthesize OC in response to $1,25(OH)_2D_3$ in a concentration-dependent manner, as compared with those maintained under normal glucose or high mannitol conditions. The effect of glucose may be mediated at the transcriptional level since the $1,25(OH)_2D_3$-induced increase in the abundance of OC mRNA was also inhibited by exposure to high glucose. The impaired cellular response to $1,25(OH)_2D_3$ may be attributable to a decrease in the number of $1,25(OH)_2D_3$ receptors. Since high mannitol, an isosmolar control of high glucose, did not mimic the effect of high glucose, the effect of high glucose was not mediated by hyperosmolarity.

Many patients with diabetes mellitus show a moderate reduction in bone mass[4-10] as a result of the altered metabolism of bone and minerals.[1-3] Proposed mechanisms were divided into two categories: high blood glucose concentrations and insulin deficiency. Hyperglycemia and glycosuria increase the loss of Ca into urine by decreasing the tubular reabsorption of Ca,[11-13] resulting in secondary hyperparathyroidism and loss of bone mineral. Alternatively, insulin deficiency may directly cause loss of bone mineral with a secondary increase in the urinary excretion of Ca and phosphorus, or indirectly by inhibiting parathyroid hormone (PTH)-induced 1α-hydroxylation of 25-hydroxyvitamin D to the active $1,25(OH)_2D$[39] and PTH secretion from the parathyroid glands.[40] The serum OC concentration in the diabetics was shown to be significantly lower than that in age- and sex-matched controls.[41] Furthermore, serum OC values were shown to be low, relative to those in control subjects, in diabetic patients receiving insulin treatment and oral antidiabetic drugs.[16] This decrease in serum OC concentration may be explained by a decrease in the serum $1,25(OH)_2D$ concentration.

Indeed a significant decrease in serum $1,25(OH)_2D$ has been demonstrated in young patients with insulin-dependent diabetes mellitus.[42] Therefore, the decreased serum levels of OC might in part be explained by a decrease of $1,25(OH)_2D$, since OC expression is intimately regulated by $1,25(OH)_2D$. However, in contrast to the patients with insulin-dependent diabetes mellitus, serum $1,25(OH)_2D$ concentrations were not significantly lower in individuals with non-insulin-dependent diabetes mellitus than in control subjects,[9,43] indicating that a decrease in serum $1,25(OH)_2D$ is not responsible for the decrease in serum OC concentration in these patients. Whereas fluoride increased serum OC concentration in controls, it had no such effect in diabetic patients receiving insulin or oral antidiabetic drug treatment.[16] Furthermore, the $1,25(OH)_2D_3$-induced increase in serum OC is less marked in diabetic BB rats than in control rats.[44]

These data support the hypothesis that deficient osteoblast function in vivo could be responsible for diabetic osteopenia. They are also consistent with the result of the present study showing an inhibitory effect of high glucose on the responsiveness of osteoblast-like cells to $1,25(OH)_2D_3$. However, the inhibitory effect of high glucose on osteoblast-like cells in vitro was less marked than the hyporesponsiveness of osteoblastic cells observed in in vivo studies. This discrepancy appears to be attributable to the insulin deficiency that accompanies the in vivo condition in insulin-

dependent diabetic humans and animals. The shorter duration of high glucose exposure in this study may also have contributed to the modest effect on osteoblast function.

We have also observed that high glucose, but not high mannitol, significantly inhibits MG-63 cell proliferation in a concentration-dependent manner (up to 55.0 mM) when the cells are plated at lower densities (Terada et al. in preparation). This effect of high glucose was prevented by the simultaneous addition of an aldose reductase inhibitor, epalrestat,[45] indicating that high glucose may inhibit osteoblast function as a result of an accumulation of sorbitol. This conclusion is further supported by the observation that high glucose-treated MG-63 cells show a markedly increased intracellular sorbitol concentration (Terada et al. in preparation), and by the demonstration of aldose reductase immunoreactivity in rat bone by immunohistochemistry.[41]

Administration of aldose reductase inhibitor to diabetic rats for 3 months failed to influence the biochemical abnormalities associated with diabetes.[41] However, the serum concentration of PTH in the diabetic rats was significantly higher than that of control rats, indicating that the development of osteopenia in these animals may be attributable mainly to negative Ca balance, as a result of increased loss of Ca into urine or decreased Ca absorption from the intestine or to a failure to gain weight, rather than to osteoblast dysfunction. Furthermore, the duration of aldose reductase inhibitor administration was not long enough to prevent diabetic complications such as nerve and eye abnormalities.[46] Therefore, the lack of a protective effect of aldose reductase inhibitor against the development of diabetic osteopenia in these experiments may not negate the possibility that the polyol pathway is important in the pathogenesis of diabetic osteopenia.

Activation of the cAMP signal transduction pathway by forskolin, dibutyryl cAMP, parathyroid hormone-related peptide, or prostaglandin E_2 results in an increase in $1,25(OH)_2D_3$ receptor mRNA abundance in the phenotypically osteoblast cell line UMR-106.[38] Furthermore, we have shown that activators of the cAMP-PKA system also cause up-regulation of $1,25(OH)_2D_3$ receptors in human promyelocytic leukemic HL-60 cells[37] and that $1,25(OH)_2D_3$ receptor number correlates positively with the intracellular cAMP concentration in HL-60 cells.[26] However, although high glucose increased the intracellular cAMP concentration, resulting in the activation of PKA in MG-63 cells, dibutyryl cAMP had no effect on $1,25(OH)_2D_3$ receptor number in these cells. Thus, the cAMP-PKA system does not appear to be important in the regulation of $1,25(OH)_2D_3$ receptor number in MG-63 cells.

In summary, our results indicate that high glucose concentrations alone significantly impair the ability of osteoblast-like cells to synthesize OC, and thus may be responsible, at least in part, for the development of diabetic osteopenia by suppressing osteoblast function.

REFERENCES

1. Schneider LE, Schedl HP 1972 Diabetes and intestinal calcium absorption in the rat. Am J Physiol **223:**1319–1323.
2. Rumenapf G, Issa S, Schwille PO 1987 The influence of progressive hyperinsulinemia on duodenal calcium absorption in the rat. Metabolism **36:**60–65.
3. Dixit PK, Ekstrom RA 1980 Decreased breaking strength of diabetic rat bone and its improvement by insulin treatment. Calcif Tissue Int **32:**195–200.
4. Albright F, Reifenstein EC 1948 Parathyroid Glands and Metabolic Bone Disease: Selected Studies. Williams and Wilkins, Baltimore, pp. 150–160.
5. Levin ME, Boisseau VC, Avioli LV 1976 Effect of diabetes mellitus on bone mass in juvenile and adult onset diabetes. N Engl J Med **294:**241–244.
6. Wu K, Schubeck KE, Frost HM 1970 Haversian bone formation rates determined by a new method in human diabetes and osteoporosis. Calcif Tissue Res **6:**204–219.
7. Hough S, Avioli LV, Bergfeld MA, Fallon MD, Slatopolsky E, Teitelbaum SL 1981 Correction of abnormal bone and mineral metabolism in chronic streptozotocin-induced diabetes mellitus in the rat by insulin therapy. Endocrinology **108:**2228–2234.
8. Okuno Y, Nishizawa Y, Sekiya K, Hagiwara S, Miki T, Morii H 1991 Total and regional bone mineral content in patients with non-insulin dependent diabetes mellitus. J Nutr Sci Vitaminol **37** (Suppl):S43–S49.
9. Imura H, Seino Y, Nakagawa S, Goto Y, Kosaka K, Sakamoto N, Kaneko T, Mimura G 1987 Diabetic osteopenia in Japanese—A geographic study. J Jpn Diabetes Soc **30:**919–924.
10. McNair P, Madsbad S, Christiansen C, Christiansen MS, Faber OK, Binder C, Transbol I 1979 Bone loss in diabetes: Effects of metabolic state. Diabetologia **17:**283–286.
11. McNair P, Madsbad S, Christiansen C 1979 Bone mineral loss in insulin treated diabetes mellitus: Studies on pathogenesis. Acta Endocrinol **90:**463–472.
12. Gallagher JC, Melton LJ, Riggs BL 1980 Examination of prevalence rates of possible risk factors in a population with a fracture of the proximal femur. Clin Orthoped **153:**158–165.
13. Raskin P, Stevenson MR, Barilla DE, Pak CY 1978 The hypercalciuria of diabetes mellitus: Its amelioration with insulin. Clin Endocrinol **9:**329–335.
14. Goodman WG, Hori T 1984 Diminished bone formation in experimental diabetes. Diabetes **33:**825–831.
15. Klein M, Frost HM 1964 The numbers of bone resorption and formation in rib. Henry Ford Hosp Med Bull **12:**527–536.
16. Rico H, Hernandez ER, Cabranes JA, Gomez-Castresana F 1989 Suggestion of a deficient osteoblastic function in diabetes mellitus: The possible cause of osteopenia in diabetics. Calcif Tissue Int **45:**71–73.
17. Brown JP, Delmas PD, Malaval L, Edouard C, Chapuy MC, Meunier PJ 1984 Serum bone Gla-protein: a specific marker for bone formation in postmenopausal osteoporosis. Lancet **i:**1091–1093.
18. Palmieri E, Pedrazzoni M, Malaquinoo AM, Carapezzi C, Carbognani A, Maroni L 1984 Osteocalcin levels in diabetes mellitus. In: Christiansen C, Arnaud CD, Parfitt AM, Peck WA, Riggs L (eds.) Osteoporosis. Aalborg Stiftsbogtrykkeri, Copenhagen, Denmark, pp. 809–810.
19. Ishida H, Seino Y, Taminato T, Usami M, Takeshita N, Seino Y, Tsutsumi C, Moriuchi S, Akiyama Y, Hara K, Imura H 1988 Circulating levels and bone contents of bone γ-carboxyglutamic acid-containing protein are decreased in streptozotocin-induced diabetes: Possible marker for diabetic osteopenia. Diabetes **37:**702–706.
20. Wettenhall REH, Schwarz PL, Bornstein J 1969 Actions of insulin and growth hormone on collagen and chondroitin sulfate synthesis in bone organ cultures. Diabetes **18:**280–284.
21. DeLuca HF, Schnoes HK 1983 Vitamin D: Recent advances. Ann Rev Biochem **52:**411–439.

22. Lajeunesse D, Kiebzak GM, Frondoza C, Sacktor B 1991 Regulation of osteocalcin secretion by human bone cells and by the human osteosarcoma cell line MG-63. Bone Miner **14:**237–250.
23. Koyama H, Nishizawa Y, Inaba M, Hino M, Prahl JM, DeLuca HF, Morii H 1994 Impaired homologous upregulation of vitamin D receptor in rats with chronic renal failure. Am J Physiol **266:**F706–F712.
24. Inaba M, DeLuca HF 1989 Stabilization of 1,25-dihydroxyvitamin D-3 receptor in the human leukemia cell line, HL-60, with diisopropylfluorophosphate. Biochim Biophys Acta **1010:** 20–27.
25. Lee Y, Inaba M, DeLuca HF, Mellon WS 1989 Immunological identification of 1,25-dihydroxyvitamin D_3 receptors in human promyelocytic leukemic cells (HL-60) during homologous regulation. J Biol Chem **264:**13701–13705.
26. Inaba M, Trinidad MB, DeLuca HF 1991 Characteristics of the 25-hydroxyvitamin D3- and 1,25-dihydroxyvitamin D3-24-hydroxylase(s) from HL-60 cells. Arch Biochem Biophys **284:** 257–263.
27. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248–254.
28. Williams D, Gorski J 1974 Equilibrium binding of estradiol by uterine cell suspensions and whole uteri in vitro. Biochemistry **13:**5537–5542.
29. Skjodt H, Gallagher JA, Beresford JN, Couch M, Poster JW, Russel RGG 1985 Vitamin D metabolites regulate osteocalcin synthesis and proliferation of human bone cells in vitro. J Endocrinol **105:**391–396.
30. Nakatsuka K, Miki T, Nishizawa Y, Tabata T, Inoue T, Morii H, Ogata E 1991 Circulating bone Gla protein in end-stage renal disease determined by newly developed two-site immunoradiometric assay. In: Morii H (ed.) Calcium Regulating Hormone, Contrib Nephrol Vol. 90, Karger, Basel, pp. 147–154.
31. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:**156–159.
32. Inaba M, Koyama H, Hino M, Okuno S, Terada M, Nishizawa Y, Nishino T, Morii H 1993 Regulation of release of hepatocyte growth factor from human promyelocytic leukemia cells, HL-60, by 1,25-dihydroxyvitamin D3, 12-*O*-tetradecanoylphorbol 13-acetate, and dibutyryl cyclic adenosine monophosphate. Blood **82:**53–59.
33. Inaba M, Okuno S, Koyama H, Ishimura E, Nishizawa Y, Morii H 1993 Effect of uremic serum on 1,25-dihydroxyvitamin D_3-induced differentiation of human promyelocytic leukemia cell, HL-60. Nephron **63:**152–158.
34. Okuno S, Inaba M, Nishizawa Y, Inoue A, Morii H 1988 Effect of tolbutamide and glyburide on cAMP-dependent protein kinase activity in rat liver cytosol. Diabetes **37:**857–861.
35. Partridge NC, Kemp BE, Livesey SA, Martin TJ 1982 Activity ratio measurements reflect intracellular activation of adenosine 3′,5′-monophosphate-dependent protein kinase in osteoblasts. Endocrinology **111:**178–183.
36. Bonewald LF, Kester MB, Schwartz Z, Swain LD, Khare A, Johnson TL, Leach RJ, Boyan BD 1992 Effects of combining transforming growth factor β and 1,25-dihydroxyvitamin D_3 on differentiation of a human osteosarcoma (MG-63). J Biol Chem **267:**8943–8949.
37. Inaba M, Okuno S, Koyama H, Nishizawa Y, Morii H 1992 Dibutyryl cAMP enhances the effect of 1,25-dihydroxyvitamin D_3 on a human promyelocytic leukemia cell, HL-60, at both the receptor and the postreceptor steps. Arch Biochem Biophys **293:**181–186.
38. van Leeuwen JP, Birkenhager JC, Vink-van Wijngaarden T, van den Bemd GJ, Pols HA 1992 Regulation of 1,25-dihydroxyvitamin D3 receptor gene expression by parathyroid hormone and cAMP-agonists. Biochem Biophys Res Commun **185:**881–886.
39. Henry HL, Norman AW 1984 Vitamin D: Metabolism and biological actions. Ann Rev Nutr **4:**493–520.
40. Sugimoto T, Ritter C, Morrissey J, Hayes C, Slatopolsky E 1990 Effects of high concentrations of glucose on PTH secretion in parathyroid cells. Kidney Int **37:**1522–1527.
41. Glajchen N, Epstein S, Ismail F, Thomas S, Fallon M, Chakrabarti S 1988 Bone mineral metabolism in experimental diabetes mellitus: osteocalcin as a measure of bone remodeling. Endocrinology **123:**290–295.
42. Frazer TE, White NH, Hough S, Santiago JV, McGee BR, Bryce G, Mallon J, Avioli LV 1981 Alterations in circulating vitamin D metabolites in the young insulin-dependent diabetics. J Clin Endocrinol Metab **53:**1154–1159.
43. Auwerx J, Dequeker J, Bouillon R, Geusens P, Nijs J 1988 Mineral metabolism and bone mass at peripheral and axial skeleton in diabetes mellitus. Diabetes **37:**8–12.
44. Verhaeghe J, Suiker AMH, Van Bree R, Van Herck E, Jans I, Visser WJ, Thomasset M, Allewaert K, Bouillon R 1993 Increased clearance of 1,25-$(OH)_2D_3$ and tissue-specific responsiveness to 1,25-$(OH)_2D_3$ in diabetic rats. Am J Physiol **265:** E215–E223.
45. Terada M, Inaba M, Yoshida O, Nishizawa Y, Otani S, Morii H 1994 Diabetic osteopenia; impaired proliferation in human osteoblast-like cell, MG-63, by high glucose. J Bone Miner Res **9** (Suppl 1):S351.
46. Tilton RG, Pugliese G, LaRose LS, Faller AM, Chang K, Province MA, Williamson JR 1991 Discordant effects of the aldose reductase inhibitor, sorbinil, on vascular structure and function in chronically diabetic and galactosemic rats. J Diabet Compl **5:**230–237.

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