Increased GABA Transport Activity in Rat Calvarial Osteoblasts Cultured under Hyperglycemic Conditions

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Several independent lines of evidence indicate the direct impairment by extracellular glucose at high concentrations of different osteoblastic functions with a marked decrease in bone mass toward osteoporosis, while the underlying mechanisms are not well clarified to date. We have previously demonstrated the functional expression of the neural amino acid γ-aminobutyric acid (GABA) signaling system including betaine/GABA transporter-I (BGT-1) with a temperature-, sodium- and chloride-dependent activity of [3H]GABA accumulation in cultured rat calvarial osteoblasts. In this study, therefore, we attempted to demonstrate the possible involvement of BGT-1 isoform in bone dysfunctions due to impaired mineralization in rat calvarial osteoblasts cultured under hyperglycemic conditions. No significant change was seen in [3H]GABA accumulation in osteoblasts cultured for 7 d in vitro (DIV) under hyperglycemic conditions (glucose=25.5–50.5 mM) compared to those cultured in normoglycemic (glucose=5.5 mM) and hyperosmotic (mannitol=25.5–50.5 mM) conditions. In osteoblasts cultured for 14 DIV under hyperglycemic conditions, however, [3H]GABA accumulation was significantly increased compared to those cultured under normoglycemic and hyperosmotic conditions. Kinetic analysis revealed that hyperglycemic cultivation resulted in a significant increase in V\textsubscript{max} values from 2.85 nmol/min/mg protein for normoglycemic conditions to 4.17 nmol/min/mg protein for hyperglycemic conditions without affecting K\textsubscript{m} values. However, experimental hyperglycemia did not significantly affect the expression of mRNA for BGT-1 isoform by osteoblasts. These results suggest that GABA transport system may at least in part play a role in pathological malfunctions and abnormalities through a mechanism not directly related to gene expression in osteoblasts under hyperglycemia.

Key words diabetic osteopenia; osteoblast; γ-aminobutyric acid (GABA) transporter; glucose

Insulin-dependent diabetes mellitus (IDDM; Type I) is characterized with low or no insulin production, which sometimes leads to diabetic osteopenia and osteoporosis.\textsuperscript{1,2,1} In the absence of insulin, insulin-sensitive cells exhibit marked reduction of glucose uptake activity, resulting in increased serum glucose levels and subsequent development of a variety of diabetic complications. Histological and bone marker assessments indicate a low turnover state in bone formation rate and decreased osteoblastic activity in rat models of type I diabetes.\textsuperscript{3,1} In addition, there is a report about the close relationship between the bone loss and the fasting blood glucose level.\textsuperscript{4} The occurrence of hyperglycemic bone loss is controversial because not only high glucose but also other factors including insulin deficiency could mediate bone loss in IDDM patients, however, while little is known about the direct effect of hyperglycemia on bone metabolism. By contrast, in vitro analysis reveals the induction by hyperglycemia of osteoblastic dysfunctions. In human osteosarcoma (MG-63) cells cultured under high glucose conditions (=55 mM), for example, impaired response is seen to parathyroid hormone and to 1,25-dihydroxyvitamin D\textsubscript{3}, an active form of vitamin D\textsubscript{3}, required for the synthesis of the matured osteoblast marker protein osteocalcin.\textsuperscript{5,6} Other reports using calvarial osteoblastic cell line MC3T3-E1 cells have demonstrated that high glucose (=15.5 mM) inhibits Ca\textsuperscript{2+} intake and bone mineralization with an increase in both cellular proliferation and alkaline phosphatase (ALP) activity.\textsuperscript{7} These findings indicate that elevated extracellular glucose concentrations would directly impair osteoblastic functions resulting in defective mineralization similar to clinical findings. Therefore, these in vitro analyses are useful for the better understanding of mechanisms relevant to osteoblastic malfunctions associated with diabetes mellitus.

On the other hand, we have previously reported the possible functional expression of particular GABAergic signaling machineries in cultured rat calvarial osteoblasts.\textsuperscript{8,9} GABA is known as one of the most abundant inhibitory amino acid neurotransmitters in the mammalian central nervous system (CNS). In the CNS, GABA is supposed to mediate inhibitory neurotransmission thorough different signaling machineries including GABA synthase, GABA receptors and GABA transporters.\textsuperscript{10–13} These GABAergic machineries are found not only in the CNS but also in some non-neuronal and peripheral organs such as heart, lung, kidney, adrenal, pancreas, liver, spleen and uterus.\textsuperscript{10} In addition to these peripheral tissues, the expression of GABA and particular GABAergic signaling molecules is found in bone cells such as osteoblasts\textsuperscript{8,9} and chondrocytes.\textsuperscript{14–16} Amongst different isoforms of GABA transporters required for signal termination, expression of betaine/GABA transporter-I (BGT-1) is exclusively detected at mRNA and protein levels with a temperature-, sodium- and chloride-dependent activity of [3H]GABA accumulation in osteoblasts, as well as GABAA receptor.\textsuperscript{8,9} Although GABAA receptor is shown to play an inhibitory role in osteoblastic differentiation and/or maturation, the role of BGT-1 isoform has not yet been well understood. In this study, therefore, we have attempted to demonstrate the possible relationship between GABA transport mediated by BGT-1 isoform and osteoblastic dysfunctions using [3H]GABA accumulation in osteoblasts cultured under hyperglycemic conditions as a cellular model for osteopenia seen under hyperglycemia.

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MATERIALS AND METHODS

Materials $\gamma$-[2,3-$^3$H(N)]-Aminobutyric acid (1480 GBq/mmol) and [3$^3$H]-Glu (1587.3 GBq/mmol) were obtained from Perkin-Elmer Life Science (Boston, MA, U.S.A.). GABA, d-glucose, d-mannitol and C-TEST kit were obtained from Wako Pure Chemicals (Osaka, Japan). Taq polymerase was purchased from Takara (Tokyo, Japan). Bio-Rad Protein Assay Kit was provided by Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other chemicals used were of the highest purity commercially available.

Preparation of Primary Cultured Osteoblasts The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques. Osteoblasts were isolated from calvaria of 1- to 2-d-old Wistar neonatal rats by the sequential enzymatic digestion method as described previously.

In brief, rat calvaria were gently incubated at 37 °C for 10 min with 0.2% (w/v) collagenase in α-modified minimum essential medium (α-MEM), followed by collection of cells in supernatants thus obtained. This incubation was consecutively repeated 5 times. Cells obtained during the last 3 digestion processes were altogether collected in α-MEM containing 10% fetal bovine serum (FBS) and several antibiotics, followed by centrifugation at 250 × g for 5 min. The pellets were suspended in α-MEM containing 10% FBS. Cells were plated at a density of 1 × 10⁶ cells/cm² in appropriate dishes, and then cultured at 37 °C for different periods under 5% CO₂, with medium change every 2 d. Throughout experiments, α-MEM containing 10% FBS, 50 μg/ml ascorbic acid and 5 mM sodium β-glycerophosphate was used.

Mineralization Analysis Osteoblasts were plated at a density of 1 × 10⁶ cells/cm² in 24-well dishes and cultured for 28 d in vitro (DIV). Alizarin red staining was performed as previously reported. In brief, cells were washed twice with cold calcium- and magnesium-free phosphate-buffered saline (PBS), followed by fixation in ice cold 70% ethanol for 1 h at 4 °C and subsequent washing with nanopure water. Cells were stained with 40 mM alizarin red-S (pH 4.2) for 10 min at 4°C and subsequent washing with nanopure water. Cells were washed twice with cold PBS, and then sonicated in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% (v/v) Triton X-100. Hydrochloric acid at 6 M was added to these cell lysates to make a final concentration of 2 M HCl. Following incubation for 16 to 24 h at room temperature, cell lysates were centrifuged at 20,000 g for 5 min, and Ca²⁺ content was determined in the supernatant using the C-TEST kit.

Determination of ALP Activity Osteoblasts were plated at a density of 1 × 10⁴ cells/cm² in 24-well dishes and cultured for different periods. Cells were washed twice with cold PBS, and then sonicated in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% (v/v) Triton X-100. The assay buffer composed of 0.05 M 2-amino-2-methylpropanol, 2 mM MgCl₂ and 10 mM p-nitrophenylphosphoric acid was added at a volume of 200 μl into 10 μl of cell suspensions, followed by a reaction for 30 min at 37 °C and subsequent immediate determination of absorbance of p-nitrophenol at 405 nm. Protein concentration was determined with a Bio-Rad Protein Assay Kit, and ALP activity was standardized on the basis of both cellular protein content and incubation time for the representation as pmol/min/μg protein.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Cultured osteoblasts were superficially washed with PBS twice, followed by extraction of total RNA using ISO-GEN (Nippon gene, Osaka, Japan) according to the manufacturer’s instructions and subsequent synthesis of complementary DNA (cDNA) with 25 ng/μl oligo (dT)₁₈ primer, 0.5 mM dNTP (deoxy nucleotide triphosphate) mix and M-MLV Reverse Transcriptase. Reverse transcriptase reaction was run at 37 °C for 50 min, and an aliquot of synthesized cDNA was directly used for PCR performed in buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM each of dNTP, 20 pmol of primers for BGT-1 isoform and 2U of Taq DNA polymerase as described elsewhere. Quantitative analysis was done with primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequence and cycling conditions used were as follows: BGT-1, (sense) 5’ AGGGAGGCCTTCCCTTCCAAG3’, (antisense) 5’ TTGGGTCCTGCAAGGCTGC3’, denaturation at 95 °C for 1 min, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min; GAPDH, (sense) 5’ GTGTAAGGTCCGGTCAACGGT3’, (antisense) 5’ GTGCCAAGTTGTCTAGGACC3’, denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Electrophoresis was run for an aliquot of PCR amplification products on a 2% agarose gel, followed by detection of DNA with ethidium bromide. Appropriate PCR products were quantified by using a densitograph with scion image for Windows, followed by calculation of ratios of expression of mRNA for BGT-1 isoform over that for GAPDH.

Determination of [3H]GABA Accumulation Osteoblasts were cultured for 7 and 14 DIV, followed by washing with HEPES Krebs–Ringer (HKR) (125 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgSO₄, 1.25 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES and 10 mM d-glucose, pH 7.4) buffer twice and subsequent further incubation in HKR buffer at 37 °C for 1 h in 5% CO₂ incubator. Cells were then incubated with 1 μM [3H]GABA at 37 °C for 20 min unless indicated otherwise. Reaction was terminated by the careful aspiration of buffer, followed by rinsing with ice-cold HKR buffer containing 1 mM unlabeled GABA at 2 °C three times and subsequent solubilization with 0.1 M NaOH for liquid scintillation spectrometry using 3 ml scintillation cocktail (clear sol I). Nonspecific accumulation was defined in parallel experiments with 1 mM unlabeled GABA in the incubation mixture and subtracted from total accumulation to calculate specific accumulation.

Data Analysis Results were expressed as the mean ± S.E. and the statistical significance was determined by Students’ t-test or one-way analysis of variance (ANOVA) with Scheffe’s F post hoc test.

RESULTS

Hyperglycemia Prevents Mineralization of Osteoblasts Marked expression was seen for Runx2 known to be a master
regulator of osteoblastic differentiation in rat calvarial osteoblasts cultured from 7 to 28 DIV (data not shown). The ALP activity, an early maker of osteoblastic differentiation, was increased in proportion to culture periods from 3 to 28 DIV with a plateau within 14 DIV in osteoblasts, while the marker protein of osteoblast maturation osteocalcin was predominantly expressed in osteoblasts cultured from 14 to 28 DIV (data not shown). Osteoblasts cultured for 28 DIV were highly stained with Alizarin red staining for mineralization (Fig. 1a, upper left panel), while a reduction was seen of Alizarin red staining intensity in osteoblasts cultured in α-MEM containing glucose at 50.5 mM, but not at 25.5 mM, for 28 DIV (Fig. 1a, upper panels). However, no marked change was seen in Alizarin red staining intensity in osteoblasts cultured in the presence of mannitol at the same concentration range. Similar results were found in von Kossa staining used for Ca²⁺ accumulation determination (data not shown). Density of Alizarin red stained area was significantly decreased in osteoblasts cultured in the presence of glucose at 50.5 mM compared to those cultured under normoglycemic (glucose = 5.5 mM) and hyperosmotic (mannitol = 25.5—50.5 mM) conditions. Consistent with the observations on Alizarin red staining, Ca²⁺ accumulation was significantly decreased in osteoblasts cultured for 28 DIV in the presence of glucose at high concentrations of 25.5 to 50.5 mM compared to normoglycemic and hyperosmotic control cells (Fig. 1b). By contrast, ALP activity was significantly increased in osteoblasts cultured in the presence of glucose at 25.5 and 50.5 mM for 14 (Fig. 1c) or 28 (Fig. 1d) DIV, compared to cells cultured under hyperosmotic control conditions. No remarkable alteration was seen with ALP activity in osteoblasts cultured for 14 and 28 DIV in the presence of mannitol at 25.5 and 50.5 mM.

[³H]GABA Accumulation Is Increased in Osteoblasts Cultured under Hyperglycemic Conditions To investigate a possible role of particular GABA transporters in osteopenia under hyperglycemia, [³H]GABA accumulation was determined in osteoblasts cultured under hyperglycemic conditions. As shown in Fig. 2a, a significant increase was induced in [³H]GABA accumulation in osteoblasts cultured in the presence of glucose at 25.5 and 50.5 mM for 14 DIV compared to that in cells cultured with mannitol at the same concentrations. No marked change was seen in [³H]GABA accumulation in osteoblasts cultured for 14 DIV in the presence of mannitol at the same concentrations. In osteoblasts cultured for 7 DIV, moreover, no significant increase was seen in [³H]GABA accumulation under hyperglycemic conditions compared to that under normoglycemic conditions (data not shown). To test the selectivity for [³H]GABA accumulation, we next determined [³H]glutamate (Glu) accumulation in osteoblasts cultured under similar experimental conditions. In contrast to [³H]GABA accumulation, however, no significant change was induced in [³H]Glu accumulation in osteoblasts cultured for 7 (data not shown) and 14 (Fig. 2b) DIV under hyperglycemic conditions. In order to evaluate the possible participation of altered cellular maturation in the increased [³H]GABA accumulation under hyperglycemia, we next investigated developmental alterations of both [³H]GABA and [³H]Glu accumulation in proportion to the duration of cultivation from 7 to 14 DIV. Throughout this culture period, [³H]Glu accumulation was 5 to 12 times higher than [³H]GABA accumulation in osteoblasts [7 DIV, 3.9±0.1 vs. 20.2±1.4; 14 DIV, 4.8±0.2 vs. 70.0±3.8 (pmol/mg protein/min)] (Fig. 2c). In proportion to cellular maturation from 7 to
14 DIV, thus, [3H]Glu accumulation was more than tripled with [3H]GABA accumulation being unchanged. Hyperglycemia Does Not Affect mRNA Expression for BGT-1 Isoform

Further kinetic analysis using different [3H]GABA concentrations revealed that hyperglycemia increased the maximal velocity of [3H]GABA accumulation in osteoblasts cultured for 14 DIV (Fig. 3a). Hanes–Woolf plot analysis of these saturation isotherms showed that [3H]GABA accumulation consisted of a single component with a $K_m$ value of 768 $\mu$M and a $V_{max}$ value of 2.85 nmol/min/mg protein, respectively, in cultured rat calvarial osteoblasts (Fig. 3b). In osteoblasts cultured under hyperglycemic conditions for 14 DIV, [3H]GABA accumulation was composed of a single component with a $K_m$ value of 930 $\mu$M and a $V_{max}$ value of 4.17 nmol/min/mg protein, respectively. To clarify the mechanism for the increased $V_{max}$ value, we next performed semi-quantitative RT-PCR analysis on mRNA expression for BGT-1 subtype expressed in osteoblasts. However, mRNA expression was not significantly affected for BGT-1 isoform in osteoblasts cultured for 14 DIV irrespective of the presence glucose and mannitol at high concentrations (Fig. 3c). Other GABA transporters not expressed in osteoblasts cultured under normal conditions were not found at an mRNA level even in osteoblasts cultured under hyperglycemic conditions on RT-PCR. These included GAT-1, GAT-2 and GAT-3 isoforms (data not shown).

DISCUSSION

This study has demonstrated that one of neural GABAergic functions such as GABA transport is significantly activated in dysfunctional osteoblasts exposed to high concentrations of glucose. The possibility that a particular GABA transporter may play a hitherto unidentified role in the pathogenesis and etiology of bone diseases related to malfunctions of osteoblasts under hyperglycemia is thus not ruled out so far.
The present findings that hyperglycemic cultivation resulted in immature morphology associated with increased ALP activity and decreased mineralization in osteoblasts, are consistent with previous reports on osteoblastic dysfunctions under hyperglycemia using osteoblastic cell line \(^7\) and in agreement with the clinical findings that serum osteocalcin levels are reduced in patients with diabetes mellitus. \(^3\) One possible speculation is that the increased GABA transport activity could be a new criterion for hyperglycemic osteopenia and/or osteoporosis. Moreover, it should be noted that hyperglycemic cultivation increased the activity of \([^{3}H]\)GABA accumulation, without significantly altering \([^{3}H]\)Glu accumulation recently identified and characterized in osteoblasts in our laboratory. \(^19\)

Of different isoforms of GABA transporters expressed in the brain, we have previously demonstrated the exclusive expression of BGT-1 isoform in primary cultured rat calvarial osteoblasts. \(^9\) The findings that no GABA transporters other than BGT-1 isoform were expressed even in osteoblasts cultured under high glucose conditions give rise to an idea that BGT-1 isoform may be highly responsible for the increased \([^{3}H]\)GABA accumulation in osteoblasts cultured under hyperglycemic conditions. Although kinetic analysis is suggestive of the upregulation of BGT-1 subtype, however, our study is unable to demonstrate a significant increase in BGT-1 mRNA expression by experimental hyperglycemia. Although BGT-1 mRNA expression was only determined in osteoblasts cultured for 14 DIV under hyperglycemic conditions, one possible explanation for this paradox is that the increased \([^{3}H]\)GABA accumulation would be mediated by a mechanism related to posttranscriptional modification of BGT-1 isoform. The activity of membrane translocation could be modulated with BGT-1 isoform under hyperglycemia. Indeed, phosphorylation by protein kinase C (PKC) or tyrosine kinase is shown to regulate the expression of GAT-1 at cell surface for subsequent functional alterations. \(^20—22\) In human BGT-1 subtype, in fact, there are two putative phosphorylation sites by PKC to modulate GABA transport activity, \(^21\) in addition to tyrosine residues in intracellular domains, which are well conserved along with those of GAT-1 subtype. \(^24\) Therefore, it is possible to speculate that BGT-1 activity might be similarly under regulation by these phosphorylation processes in a particular situation.

Hyperosmolarity is shown to modulate the activity of BGT-1 isoform, \(^25\) meanwhile, while our data are clearly unfavorable for the possible participation of hyperosmolarity in the increased \([^{3}H]\)GABA accumulation under high glucose conditions. Taken together, glucose rather than osmolarity seems to be really responsible for the increased \([^{3}H]\)GABA accumulation activity in osteoblasts cultured under hyperglycemic conditions. Hyperglycemia induces the inhibition of mineralization with a concomitant increase in ALP activity in osteoblasts as shown in this study, \(^7\) but underlying mechanisms have not yet been clarified to date. A possible interpretation for the increased \([^{3}H]\)GABA accumulation is that hyperglycemia would disturb normal cellular maturation to lead to malfunctions of amino acid transporters expressed by osteoblasts. However, our present results on developmental changes in both \([^{3}H]\)GABA and \([^{3}H]\)Glu accumulation give rise to an idea that the increased GABA transport activity may be at least in part brought about by high glucose, but not by general disturbance of cellular maturation such as differentiation in osteoblasts cultured under hyperglycemia.

The findings cited above argue in favor of a possible role of the GABA transporter BGT-1 in osteoblastic dysfunctions seen under hyperglycemia. Future studies on modulation by BGT-1 isoform of osteoblastic differentiation and maturation might provide a better understanding to the development and prevention of osteopenia and osteoporosis associated with diabetic mellitus in human beings.

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