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Enhanced and suppressed mineralization by acetoacetate and β-hydroxybutyrate in osteoblast cultures

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ABSTRACT

It is known that diabetes aggravates alveolar bone loss associated with periodontitis. While insulin depletion increases the blood concentration of ketone bodies, i.e., acetoacetate and β-hydroxybutyrate, their roles in bone metabolism have not been much studied until today. We investigated the effects of acetoacetate and β-hydroxybutyrate on mineralization of extracellular matrix in cultures of mouse osteoblastic MC3T3-E1 cells and primary mouse osteoblasts in the presence and absence of bone morphogenetic protein-2. Acetoacetate potentiated alkaline phosphatase activity in MC3T3-E1 cells in a concentration-dependent manner, ranging from physiological to pathological concentrations (0.05–5 mmol/L). In contrast, β-hydroxybutyrate lowered it in the same experimental settings. Mineralization in cultures of these cells was also up-regulated by acetoacetate and down-regulated by β-hydroxybutyrate. Similar results were obtained in cultures of mouse primary osteoblasts. Neither alkaline phosphatase mRNA nor its protein expression in MC3T3-E1 cells was affected by acetoacetate or β-hydroxybutyrate, indicating that these ketone bodies control the enzyme activity of alkaline phosphatase in osteoblasts and hence their mineralization bi-directionally. Finally, either gene silencing of monocarboxylate transporter-1, a major transmembrate transporter for ketone bodies, nullified the effects of ketone bodies on alkaline phosphatase activity in MC3T3-E1 cells. Collectively, we found that ketone bodies bidirectionally modulates osteoblast functions, which suggests that ketone bodies are important endogenous factors that regulate bone metabolism in both physiological and pathological situations.

1. Introduction

It is believed that chronic periodontitis and diabetes mellitus are deeply linked each other. Diabetes mellitus is one of the major risk factors for periodontitis, and the converse is also true [1]. In addition, diabetes aggravates alveolar bone loss associated with periodontitis [2]. In general, diabetic patients have many complications including increased fracture risk and delayed fracture healing [3–6]. While bone quality is declined through degeneration of extracellular matrix proteins by advanced glycation end products [5], it is known that bone mineral density remains unchanged or rather increases in patients with type 2 diabetes [7–9]. In addition, mineralization of muscular middle layer of the arterial walls is frequently observed in diabetic patients [10,11]. Hence it is suggested that biological mineralization is affected by some factors associated with diabetes.

In healthy individuals with good nutritional status, glucose is used as a major energy source. Pyruvate, the final product of glycolysis, is converted into acetyl CoA, which is then degraded via citric acid cycle, resulting in production of ATP by oxidative phosphorylation. Acetyl CoA is also produced by β-oxidation of fatty acids. In the liver, acetyl CoA is further metabolized into ketone bodies, namely, acetoacetate, β-hydroxybutyrate, and acetone. While acetone is excreted in expired breath, acetoacetate and β-hydroxybutyrate are transported to extra-hepatic tissues via the bloodstream, and physiological concentrations of acetoacetate and β-hydroxybutyrate in blood range from 10 to 70 μmol/L and 80–90 μmol/L, respectively [12,13].

In diabetic patients, glucose metabolism is potently suppressed

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and fatty acids are instead used as the major energy source. However, acetyl CoA produced by β-oxidation is not metabolized efficiently by citric acid cycle in diabetic patients, but preferentially metabolized into ketone bodies. The blood concentrations of acetoacetate and β-hydroxybutyrate can reach up to several mmol/L in type 1 diabetic patients [14–16].

Up to the present, the role of ketone bodies in bone metabolism has not been documented. In this study, we investigated the effects of acetoacetate and β-hydroxybutyrate on functions of mouse osteoblasts, especially the activity of alkaline phosphatase (ALP), which is essential for mineralization of extracellular matrix.

2. Materials and methods

2.1. Ketone bodies

We prepared sodium acetoacetate immediately before use by alkaline hydrolysis of ethyl acetoacetate (Wako Pure Chemicals, Osaka, Japan) according to a previously reported method [17]. Briefly, ethyl acetoacetate (0.65 mL) and 2 mol/L NaOH (2.0 mL) were mixed in pure water (22.35 mL) and incubated for 1 h at 40 °C. This reaction mixture was used as an aqueous solution of acetoacetate (0.2 mol/L). DL-β-Hydroxybutyric acid sodium salt was purchased from Sigma–Aldrich (St. Louis, MO, USA), These ketone bodies were diluted to appropriate concentrations with α-modified minimal essential medium (αMEM, Wako) supplemented with 10% fetal bovine serum (FBS, Invitrogen Co., Carlsbad, CA), and antibiotics (penicillin and streptomycin), and an antimycotic (amphotericin B).

2.2. Cell cultures

We purchased MC3T3-E1 cells, a cell line derived from newborn mouse calvarial osteoblasts, from the Riken BioResource Center (Tsukuba, Japan). We also used mouse primary osteoblasts isolated from the calvaria of newborn ddY mice provided by Japan SLC Inc. (Hamamatsu, Japan) using a conventional method [18]. Both MC3T3-E1 cells and mouse primary osteoblasts were cultured in αMEM supplemented with 10% FBS, penicillin, streptomycin, and amphotericin B at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Introduction of siRNA

A Stealth™ siRNA for mouse monocarboxylate transporter (MCT)-1 (*Mct1*) and its control non-silencing siRNA were purchased from Invitrogen. The sense and antisense sequences of *Mct1* siRNA are 5′-GGU CUU GGG CUU GCU UUC AAC UUG A-3′ and 5′-UCA AGU UGA AAG CAA GCC CAA GAC C-3′, respectively. The siRNAs (30 pmol) were introduced into MC3T3-E1 cells in antibiotic/antimycotic-free αMEM containing 10% FBS using Lipofectamine™ RNAiMAX (Invitrogen) by reverse transfection. After 24 h of incubation, the culture medium was changed to fresh one containing antibiotics/antimycotic. Reduced expression of *Mct1* mRNA was confirmed by reverse transcription (RT)-polymerase chain reaction (PCR).

2.4. ALP activity staining

Cells were plated in 96-well plates (2.0×10^4 cells/well) and cultured overnight, and further cultured for 24, 48 and 72 h in the presence or absence of 100 ng/mL recombinant human bone morphogenetic protein-2 (BMP-2, R&D Systems, Minneapolis, MN) with various concentrations of acetoacetate or DL-β-hydroxybutyrate. The cells were fixed for 30 min in 4% paraformaldehyde, washed with PBS, incubated for 30 min at 37 °C with 100 mmol/L Tris buffer (pH 8.5) containing 270 μmol/L naphthol AS-MX phosphate (Sigma–Aldrich) and 1.4 mmol/L Fast blue BB (Sigma–Aldrich), and observed under a microscope.

2.5. Determination of ALP activity

Cells were plated in 96-well plates (2.0×10^4 cells/well) and cultured overnight, and further cultured for 24, 48 and 72 h in the presence and absence of acetoacetate, DL-β-hydroxybutyrate, and BMP-2. Then the cells were washed with PBS and homogenized with 1% Nonidet P-40 (50 μL) under sonication on ice. Cell lysates (10 μL) were added to 50 μL of 0.2 mol/L Tris–HCl buffer (pH 9.5) containing 1 mmol/L $MgCl_2$ and 12.5 mmol/L disodium *p*-nitrophenyl phosphate (Wako). After incubation for 15 min at 37 °C, reactions were terminated by addition of 50 mL of 0.5 mol/L NaOH, and absorbance of the reaction mixtures at 405 nm was read using a micro-plate reader (SH-1000, Corona Electric, Ibaraki, Japan). Increase in absorbance in 15 min was divided by the amount of cellular protein to express specific activity of ALP.

2.6. Alizarin red staining

Cells were plated in 96-well plates (2.0×10^4 cells/well) and cultured overnight in αMEM plus 10% FBS and antibiotics/antimycotic, and additionally cultured for 7 days in that medium supplemented with 10 mmol/L disodium β-glycerophosphate (Sigma–Aldrich), 10 nmol/L dexamethasone (Wako), 50 μg/mL sodium L-ascorbate (Wako), and various concentrations of acetoacetate and DL-β-hydroxybutyrate in the presence and absence of 100 ng/mL BMP-2. The medium was changed every 2 days. Cells were washed twice with PBS, fixed with 95% methanol, and stained with 1% alizarin red S (Wako), then washed several times with pure water, and observed under a microscope. To evaluate the amount of calcium deposits, alizarin red S was dissolved in 10% cetylpyridinium chloride and absorbance at 570 nm was measured.

2.7. Cell viability assay

Cells were plated in 96-well plates (0.5×10^4 cells/well) and cultured overnight in αMEM supplemented with 10% FBS, antibiotics, and antimycotic, and cultured further for 24, 48, and 72 h in the media containing various concentrations of acetoacetate and DL-β-hydroxybutyrate. Cell viability was assessed using a Cell Titer 96® Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI, USA).

2.8. Statistical analysis

All data are expressed as the mean ± SD. Statistical comparisons between two and among multiple groups were conducted with a Mann–Whitney *U*-test and the Boneferroni-Holm method, respectively. Differences were considered statistically significant when *p*-values or adjusted *p*-values were lower than 0.05.

3. Results

3.1. Ketone bodies modulated ALP activity in cells in concentration- and time-dependent manners

ALP activity in MC3T3-E1 cells cultured for 3 days in the presence of BMP-2 was up-regulated by acetoacetate and down-regulated by DL-β-hydroxybutyrate in concentration-dependent manners (Fig. 1A and B). The specific activity of ALP in the cells increased in a time-dependent manner after treatment with BMP-

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