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High extracellular Ca^{2+} enhances the adipocyte accumulation of bone marrow stromal cells through a decrease in cAMP

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ABSTRACT

Bone marrow stromal cells (BMSCs) are common progenitors of both adipocytes and osteoblasts. We recently suggested that increased $[Ca^{2+}]_o$ caused by bone resorption might accelerate adipocyte accumulation in response to treatment with both insulin and dexamethasone. In this study, we investigated the mechanism by which high $[Ca^{2+}]_o$ enhances adipocyte accumulation.

We used primary mouse BMSCs and evaluated the levels of adipocyte accumulation by measuring Oil Red O staining. CaSR agonists (both Ca^{2+} and Sr^{2+}) enhanced the accumulation of adipocytes among BMSCs in response to treatment with both insulin and dexamethasone. We showed that high $[Ca^{2+}]_o$ decreases the concentration of cAMP using ELISA. Real-time RT-PCR revealed that increasing the intracellular concentration of cAMP (both chemical inducer (1 μM forskolin and 200 nM IBMX) and a cAMP analog (10 μM pCPT-cAMP)) suppressed the expression of PPARγ and C/EBPα. In addition, forskolin, IBMX, and pCPT-cAMP inhibited the enhancement in adipocyte accumulation under high $[Ca^{2+}]_o$ in BMSCs. However, this inhibited effect was not observed in BMSCs that were cultured in a basal concentration of $[Ca^{2+}]_o$. We next observed that the accumulation of adipocytes in the of bone marrow of middle-aged mice (25–40 weeks old) is higher than that of young mice (6 weeks old) based on micro CT. ELISA results revealed that the concentration of cAMP in the bone marrow mononuclear cells of middle-aged mice is lower than that of young mice. These data suggest that increased $[Ca^{2+}]_o$ caused by bone resorption might accelerate adipocyte accumulation through CaSR following a decrease in cAMP.

1. Introduction

The bone marrow stroma contains both osteoblasts and adipocytes, which have a common precursor: the pluripotent mesenchymal stem cell found in bone marrow stromal cells (BMSCs) [1–3]. High levels of marrow adipocytes are a risk factor for anemia [4] and fractures [5]. Local bone marrow Ca^{2+} levels can increase to high concentrations due to bone resorption [6], which is one of the notable features of the bone marrow stroma. We have reported that increased $[Ca^{2+}]_o$ caused by bone resorption might accelerate adipocyte accumulation rather than osteoblastic bone formation in aging and/or diabetic patients [7,8]. However, the molecular mechanisms by which high $[Ca^{2+}]_o$ may mediate an increase in the adipogenic induction of BMSCs remain unclear.

Extracellular calcium-sensing receptor (CaSR) is a G-protein coupled receptor that senses the extracellular levels of Ca^{2+}. CaSR has been reported to regulate both cell proliferation and cell differentiation of many cells [9]. Using primary mouse BMSCs, we show that CaSR agonists enhance the proliferation of BMSCs and the adipocyte differentiation of BMSCs, both of which results in the accumulation of adipocytes. The activation of CaSR increases $[Ca^{2+}]_i$ and diacylglycerol through Gaq type G proteins and decreases cAMP through Gai type G proteins. We show here that high Ca^{2+} enhances the proliferation of BMSCs through increased $[Ca^{2+}]_i$, which results in the accumulation of adipocytes. We also show that high Ca^{2+} enhances adipocyte differentiation through suppression of cAMP, which results in the

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accumulation of adipocytes. In addition, increased cAMP suppresses the enhancement of adipocyte accumulation caused by high Ca^{2+}. We also indicate that there is a correlation between the accumulation of adipocytes in the bone marrow and the decreased concentration of cAMP in vivo. We propose that the prevention of CaSR signaling, especially the cAMP-dependent pathway, could be a new therapeutic target to suppress the accumulation of adipocytes in the bone marrow and to reduce disease- and age-related anemia and fractures.

2. Materials and methods

2.1. Cell culture

The cell culture methods used in this study were described previously [10,11]. Briefly, male C57Bl/6 mice (Charles River Japan, Kanagawa, Japan) were euthanized by cervical dislocation, and bone marrow cells were collected from the tibia and femur and cultured at 37 °C in 5% CO_2/95% air. We selectively maintained adherent cells (BMSCs) by removing the non-adherent cells when changing the medium. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experimental protocol was approved by the Animal Care and Use Committee of Juntendo University. We used 10 μg/ml insulin (Sigma-Aldrich, MO, USA) and 0.2 μM dexamethasone (Sigma-Aldrich) to induce adipocyte differentiation.

2.2. Measurement of adipocyte accumulation

For Oil Red O staining and extraction, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and then rinsed twice with PBS. The cells were first treated with 60% isopropanol for 1 min and then for 20 min with Oil Red O (Sigma-Aldrich) that was dissolved in 60% isopropanol. This process was followed by three rinses with PBS. Pictures of the cells were taken, and the Oil Red O dye in lipid droplets was eluted into isopropanol. The absorbance at 520 nm was then measured with a microtiter plate reader.

2.3. Measurement of cell numbers

Cells were seeded onto 96-well plates at 5×10^3 cells per well. After 14 days, the cell counts were estimated using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay [12], which is a modification of the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) assay. In brief, 100 μL of medium and 10 μL of WST-8 reagent (Dojindo, Kumamoto, Japan) were added to the wells. After 4 h of incubation at 37 °C, the absorbance at 450 nm was recorded with a microtiter plate reader.

2.4. Quantitative real-time RT-PCR analysis

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The cDNA was then amplified using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (both from Applied Biosystems). TaqMan probes and primers (all from Applied Biosystems) for CCAAT-enhancer binding protein α (C/EBPα, assay identification number Mm00514283_s1), peroxisome proliferator-activated receptor γ (PPARγ, assay identification number Mm01184322_m1), CaSR (assay identification number Mm00443375_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assay identification number Mm99999915_g1) were used. The PCR mixtures were pre-incubated at 50 °C for 2 min, which was followed by an incubation at 95 °C for 20 s and then 40 cycles of 95 °C for 3 s and 60 °C for 30 s using the Applied Biosystems 7500 Fast real-time PCR system. The real-time data were analyzed using the 7500 software (Applied Biosystems).

2.5. Measurement of intracellular free calcium concentrations

Cells were plated onto glass-bottom dishes and loaded with 5 μM fura-2 acetoxymethylester (AM) (Dojindo) suspended in balanced salt solution (BSS) containing 125 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM glucose (the pH was adjusted to 7.4 with NaOH, 284.8 ± 2.9 mOsm/kgH_2O) in the dark for 60 min at 37 °C. The loaded cells were rinsed three times in BSS, and data acquisition and analysis were performed using AquaCosmos 2.0 (Hamamatsu Photonics, Hamamatsu, Japan). Solutions were superfused at a rate of 2 mL/min.

2.6. Measurement of intracellular concentrations of cAMP

To analyze the effect of $CaCl_2$ on cAMP concentration of BMSCs, cells were treated with 9 mM $CaCl_2$ for 10 min. To compare the cAMP concentration of bone marrow cells of young mice (6 weeks old) and middle-aged mice (25–40 weeks old), bone marrow mononuclear cells were obtained using the Ficoll-Paque protocol [13] from the tibia and femur of male C57Bl/6 mice. Intracellular concentrations of cAMP were determined using a commercial ELISA kit (Cat # 581001, Cayman Chemical Company, MI, USA) according to the protocol provided by the manufacturer.

2.7. Quantification of bone marrow adiposity

Micro-CT scanning of the tibia and femur was performed with a Latheta LCT-200 CT scanner (Hitachi Aloka Medical, Tokyo, Japan). For bone marrow adiposity imaging, mice under deep anesthesia or isolated bone were scanned with the micro-CT scanner with optimized scan energy and voxel size.

2.8. Osmolarity measurements

The osmolarity of the solutions was measured using a FISKE ONE-TEN OSMOMETER (John Morris Scientific, Sydney, NSW, Australia) according to the protocol provided by the manufacturer.

2.9. Statistical analysis

The data are expressed as the mean ± standard deviation (SD). Homogeneity of variances and mean values were confirmed with a Bartlett test and a one-way ANOVA, respectively. Significance was evaluated with a Tukey's post hoc test, and differences were considered to be significant when $P < 0.05$.

3. Results

3.1. CaSR agonists enhance the accumulation of adipocytes in BMSCs

BMSCs express CaSR. We also observed the expression of CaSR using real-time RT-PCR. We cultured BMSCs with insulin and dexamethasone for 14 days to induce differentiation into adipocytes, and the value of adipocyte accumulation was assessed using Oil Red O staining and extraction. The addition of the CaSR agonists (both $CaCl_2$ and $SrCl_2$) enhanced the accumulation of adipocytes (Fig. 1A and B). However, the addition of $MgCl_2$ did not affect the accumulation of adipocytes (Fig. 1A and B). The addition of chloride increased the osmolarity of each medium, but there were no significant differences caused by the addition of $CaCl_2$, $SrCl_2$ or $MgCl_2$ (i.e., no significant differences between groups A and B, Table 1). These results indicate that CaSR agonists rather than Cl^- or osmolarity enhance the accumulation of adipocytes. In the process of the accumulation of bone marrow adipocytes, two important factors are adipocyte differentiation and the proliferation of