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Enhanced accumulation of adipocytes in bone marrow stromal cells in the presence of increased extracellular and intracellular [Ca^{2+}]

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ABSTRACT

The bone marrow stroma contains osteoblasts and adipocytes that have a common precursor: the pluripotent mesenchymal stem cell found in bone marrow stromal cells (BMSCs). Local bone marrow Ca^{2+} levels can reach high concentrations due to bone resorption, which is one of the notable features of the bone marrow stroma. Here, we describe the effects of high $[Ca^{2+}]_o$ on the accumulation of adipocytes in the bone marrow stroma. Using primary mouse BMSCs, we evaluated the level of adipocyte accumulation by measuring Oil Red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity. High $[Ca^{2+}]_o$ enhanced the accumulation of adipocytes following treatment with both insulin and dexamethasone together but not in the absence of this treatment. This enhanced accumulation was the result of both the accelerated proliferation of BMSCs and their differentiation into adipocytes. Using the fura-2 method, we also showed that high $[Ca^{2+}]_o$ induces an increase in $[Ca^{2+}]_i$. An intracellular Ca^{2+} chelator suppressed the enhancement in adipocyte accumulation due to increased $[Ca^{2+}]_o$ in BMSCs. These data suggest a new role for extracellular Ca^{2+} in the bone marrow stroma: increased $[Ca^{2+}]_o$ induces an increase in $[Ca^{2+}]_i$ levels, which in turn enhances the accumulation of adipocytes under certain conditions.

1. Introduction

The bone marrow stroma contains both osteoblasts and adipocytes that have a common precursor: the pluripotent mesenchymal stem cell found in bone marrow stromal cells (BMSCs) [1–3]. It is well known that adipocyte accumulation is abnormally enhanced in the bone marrow stroma in the elderly or under osteoporotic conditions and also following chronic treatments of the peroxisome proliferator-activated receptor γ (PPARγ) agonist against diabetes. High levels of marrow adipocytes are a risk factor for anemia and fracture. Local bone marrow Ca^{2+} levels can reach high concentrations due to bone resorption [4], which is one of the notable features of the bone marrow stroma. High $[Ca^{2+}]_o$ enhances the chemotaxis [5,6], proliferation [5,7], and differentiation [8,9] of osteoblasts for the purposes of regenerating lost bone. However, the effects of high $[Ca^{2+}]_o$ on the accumulation of adipocytes in the bone marrow stroma are poorly understood.

Using primary mouse BMSCs, we investigated the effects of high $[Ca^{2+}]_o$ on adipocyte accumulation under conditions that result in infrequent or predominant adipocyte differentiation. We also investigated the role of high $[Ca^{2+}]_o$ -induced calcium signaling in the accumulation of adipocytes. Under the condition producing infrequent adipocyte differentiation, high $[Ca^{2+}]_o$ enhanced the accumulation of osteoblasts but not of adipocytes. Under the condition producing predominant adipocyte differentiation, high $[Ca^{2+}]_o$ enhanced the accumulation of adipocytes but not of osteoblasts. These results suggest that increased extracellular concentrations of Ca^{2+} may accelerate a switch in cell fate: increased $[Ca^{2+}]_o$ enhances osteoblastic bone formation in normal bone where adipocyte differentiation is infrequent, while increased $[Ca^{2+}]_o$ may enhance the accumulation of adipocytes in pathological bone conditions such as osteoporosis or in the elderly in which adipocyte differentiation becomes dominant. We also propose that the prevention of calcium signaling could be a new therapeutic strategy to suppress bone marrow accumulation of adipocytes and reduce disease- and age-related anemia and fracture.

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2. Materials and methods

2.1. Cell culture

The methods used for cell culture have been 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 were cultured at 37 °C in 5% CO_2/95% air. We selectively maintained adherent cells (BMSCs) by removing the floating cells during a change in medium. The 3T3-L1 pre-adipocyte cell line (3T3-L1 cells) was cultured in Dulbecco's modified Eagle's Medium (DMEM, Invitrogen, NY, USA) containing 10% fetal bovine serum (FBS, Equitech-Bio, TX, USA) at 37 °C in 5% CO_2/95% air. 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), 0.25 μM dexamethasone (Sigma–Aldrich), and 50 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma–Aldrich) as inducers of adipocyte differentiation and *O*,*O'*-Bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N'*,*N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM, Dojindo, Kumamoto, Japan) as an intracellular Ca^{2+} chelator.

2.2. Measurements of adipocyte and osteoblast accumulation

For Oil Red O staining and extraction, cells were rinsed twice with PBS and fixed with 4% paraformaldehyde for 15 min, which was followed by two rinses with PBS. The cells were first treated with 60% isopropanol for 1 min and subsequently with Oil Red O (Sigma–Aldrich) dissolved in 60% isopropanol for 20 min followed by three rinses with PBS. Pictures of the cells were taken, and Oil Red O dye in lipid droplets was eluted into isopropanol. Finally, the absorbance at 520 nm was measured with a microtiter plate reader. To measure glycerol-3-phosphate dehydrogenase (GPDH) activity, we used a GPDH activity assay kit (Takara Bio, Shiga, Japan). To measure alkaline phosphatase (ALP) activity, we used an ALP activity assay kit (Takara Bio).

2.3. 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 115 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) in the dark for 30 min. The loaded cells were rinsed three times in BSS, and data acquisition and analysis was carried out using AQUACOSMOS 2.0 (Hamamatsu Photonics, Hamamatsu, Japan). Solutions were superfused at a rate of 2 ml/min.

2.4. Measurement of cell numbers

Cells were seeded onto 96-well plates at 5×10^3 cells/well. After 14 days, the cell count was 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) were added to the wells. After a 4 h incubation at 37 °C, the absorbance at 450 nm was recorded with a microtiter plate reader.

2.5. 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). The TaqMan probes and primers (all from Applied Biosystems) for pre-adipocyte factor-1 (Pref-1, assay identification number Mm00494477_m1), CCAAT-enhancer binding protein α (C/EBPα, assay identification number Mm00514283_s1), PPARγ (assay identification number Mm01184322_m1), GPDH (assay identification number Mm00439082_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 followed by at 95 °C for 20 s and 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).

2.6. Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Homogeneity of variances and mean values were confirmed by a Bartlett test and one-way ANOVA, respectively. Significance was evaluated by Tukey's post hoc test, with differences considered significant at $P < 0.05$.

3. Results

3.1. Bone marrow stromal cells require insulin and dexamethasone to differentiate into adipocytes

First, we determined the conditions under which BMSCs differentiate into adipocytes. BMSCs were cultured in standard medium with combinations of insulin, dexamethasone, and IBMX used to induce adipocyte differentiation from the 3T3-L1 pre-adipocyte cell line (3T3-L1 cells), which is widely used as an adipocyte differentiation model. After 14 days, the value of adipocyte differentiation was assessed by staining for lipid accumulation and extraction with Oil Red O (Fig. 1A). Neither insulin, dexamethasone, nor IBMX alone induced adipocyte differentiation; in contrast, the combination of insulin and dexamethasone was capable of inducing adipocyte differentiation. We did not observe additional adipocyte differentiation in cultures containing insulin, dexamethasone, and IBMX together. Therefore, treatment with insulin and dexamethasone (differentiation medium) was used to induce adipocyte differentiation in the subsequent experiments. In contrast with BMSCs, 3T3-L1 cells required insulin, dexamethasone, and IBMX to differentiate into adipocytes (data not shown).

3.2. High $[Ca^{2+}]_o$ enhances the accumulation of adipocytes from bone marrow stromal cells

We cultured BMSCs in differentiation medium containing different $[Ca^{2+}]_o$ (1.8 mM; basal, 5.4 and 10.8 mM; high concentration), and the value of adipocyte accumulation was assessed after 14 days. Lipid accumulation was measured by Oil Red O staining and extraction, and the activity of GPDH, which is the activity of a key enzyme for lipid synthesis, was evaluated.

The high $[Ca^{2+}]_o$ group of BMSCs showed significant increases in both lipid accumulation (Fig. 1B-top, C-left) and GPDH activity (Fig. 1D-left). Furthermore, both the percentage of lipid accumulation and GPDH activity in the cells was increased in the high $[Ca^{2+}]_o$

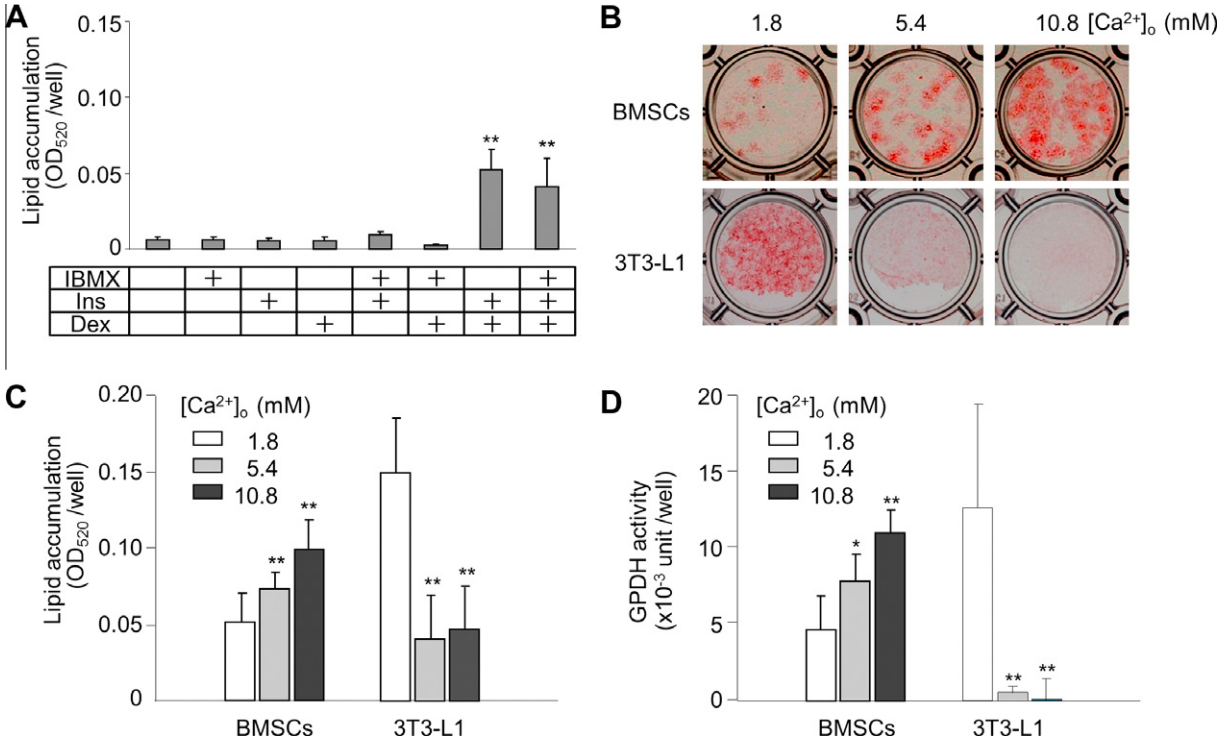

Fig. 1. High $[Ca^{2+}]_o$ enhances the accumulation of adipocytes from bone marrow stromal cells. (A) Bone marrow stromal cells (BMSCs) were cultured in standard medium with the indicated combinations of insulin (Ins), dexamethasone (Dex), and 3-isobutyl-1-methylxanthine (IBMX). After 14 days, the level of adipocyte accumulation was measured by Oil Red O staining and extraction. n = 3 (** $P < 0.01$ vs. control). (B-D) BMSCs were cultured in differentiation medium (insulin + dexamethasone) for 14 days treated with the indicated $[Ca^{2+}]_o$. (B) Typical photomicrographs of Oil Red O staining of cells are shown. The level of adipocyte accumulation was measured by Oil Red O extraction (C) and glycerol-3-phosphate dehydrogenase (GPDH) activity (D). n = 6–10 (* $P < 0.05$, ** $P < 0.01$ vs. 1.8 mM of $[Ca^{2+}]_o$ group).

group (data not shown). In contrast, the high $[Ca^{2+}]_o$ group of 3T3-L1 cells showed a significant decrease in both lipid accumulation (Fig. 1B-bottom, C-right) and GPDH activity (Fig. 1D-right). These results suggest that high $[Ca^{2+}]_o$ in BMSCs enhances adipocyte accumulation.

3.3. High $[Ca^{2+}]_o$ enhances both proliferation and adipocyte differentiation in bone marrow stromal cells

In the accumulation of bone marrow adipocytes, two important keys are adipocyte differentiation and the proliferation of BMSCs, which have the potential to differentiate into adipocytes. Next, we assessed the effects of high $[Ca^{2+}]_o$ on the proliferation of BMSCs. A total of 5×10^3 cells were seeded, and after 14 days of culture in differentiation medium, the cell counts were evaluated using a modification of the MTT assay; significant increases in the cell counts were observed for the high $[Ca^{2+}]_o$ group (Fig. 2A).

Adipocyte differentiation occurs in stages during which stage-specific adipocyte markers appear. We used real-time quantitative RT-PCR to determine whether high $[Ca^{2+}]_o$ alters the expression levels of Pref-1 (also known as delta-like homolog 1 (DLK-1), a marker of pre-adipocytes), C/EBPα and PPARγ (two important adipogenic transcription factors), and GPDH (a later marker of adipocyte differentiation) in BMSCs. One day following treatment with differentiation medium, a significant increase in the mRNA levels of Pref-1 (Fig. 2Ba), C/EBPα (Fig. 2Bb), PPARγ (Fig. 2Bc), and GPDH (Fig. 2Bd) was observed (compared to treatment with standard medium, $P < 0.01$). Differences in $[Ca^{2+}]_o$ did not affect the mRNA levels of Pref-1 (Fig. 2Ba). In contrast, the high $[Ca^{2+}]_o$ group showed a significant increase in the mRNA levels of C/EBPα (Fig. 2Bb), PPARγ (Fig. 2Bc), and GPDH (Fig. 2Bd). These results suggest that high $[Ca^{2+}]_o$ enhances both the proliferation and adipocyte differentiation of BMSCs.

3.4. An intracellular Ca^{2+} chelator suppresses the enhancement in adipocyte accumulation from bone marrow stromal cells resulting from increased $[Ca^{2+}]_o$

We used the fura-2 method to examine whether a change in $[Ca^{2+}]_o$ alters $[Ca^{2+}]_i$ levels in cells. Most of the BMSCs showed an increase in $[Ca^{2+}]_i$ in response to increased $[Ca^{2+}]_o$ (Fig. 3A, C, E). This increase in $[Ca^{2+}]_i$ was sustained during the period of increased $[Ca^{2+}]_o$ (over 30 min, Fig. 3A). In contrast, a change in $[Ca^{2+}]_i$ was not observed in 3T3-L1 cells (Fig. 3B, D).

We next examined whether intracellular Ca^{2+} affects adipocyte accumulation. We cultured BMSCs in differentiation medium for 14 days along with treatment with an intracellular Ca^{2+} chelator, BAPTA-AM, and evaluated the resulting lipid accumulation. BAPTA-AM did not affect adipocyte accumulation in BMSCs cultured at basal $[Ca^{2+}]_o$ (Fig. 3F-left, G-left). In contrast, BAPTA-AM suppressed adipocyte accumulation in BMSCs cultured with high $[Ca^{2+}]_o$ (Fig. 3F-right, G-right). These results suggest that high $[Ca^{2+}]_o$ enhances adipocyte accumulation in BMSCs through increases in $[Ca^{2+}]_i$.

3.5. The effects of high $[Ca^{2+}]_o$ in bone marrow stromal cells differ under different culture conditions

Many researchers have reported that increased bone marrow Ca^{2+} enhances the differentiation of osteoblasts. Therefore, we assessed whether our results are in agreement with previous reports. We cultured BMSCs in standard and differentiation media treated with different levels of $[Ca^{2+}]_o$. After 14 days, we assessed the

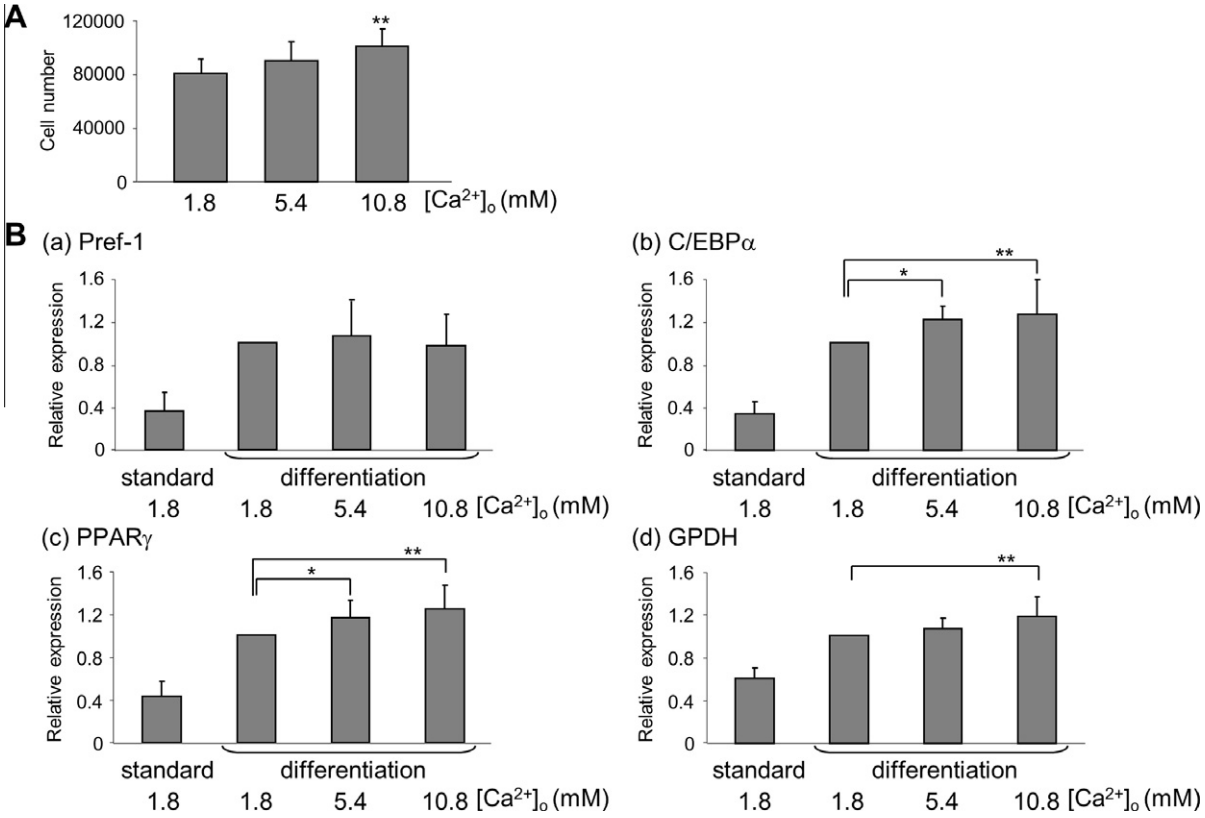

Fig. 2. High $[Ca^{2+}]_o$ enhances both the proliferation and adipocyte differentiation of bone marrow stromal cells. (A) 5×10^3 BMSCs were cultured in differentiation medium with the indicated $[Ca^{2+}]_o$. After 14 days, the cell counts were evaluated using a modification of the MTT assay. n = 9 (** $P < 0.01$ vs. 1.8 mM of $[Ca^{2+}]_o$ group). (B) BMSCs were cultured in standard medium. After 7 days, the cells were cultured for 1 more day in standard medium or differentiation medium treated with the indicated $[Ca^{2+}]_o$. Total RNA was isolated, and the quantitative mRNA levels of pre-adipocyte factor-1 (Pref-1, a), CCAAT-enhancer binding protein α (C/EBPα, b), peroxisome proliferator-activated receptor γ (PPARγ, c), and GPDH (d) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using real-time quantitative RT-PCR. n = 8 (* $P < 0.05$, ** $P < 0.01$ vs. 1.8 mM of $[Ca^{2+}]_o$ group cultured in differentiation medium).

levels of adipocyte and osteoblast accumulation using Oil Red O staining and an ALP activity assay, respectively.

The high $[Ca^{2+}]_o$ group cultured in differentiation medium (Fig. 4A-right) showed a significant increase in the number of Oil Red O-stained cells, but this increase was not observed in standard medium (Fig. 4A-left). In contrast, the high $[Ca^{2+}]_o$ group under standard medium (Fig. 4B-left) showed a significant increase in ALP activity, which was not observed in cultures grown in differentiation medium (Fig. 4B-right).

These results suggest that under certain conditions such as treatment with both insulin and dexamethasone (i.e., a condition of predominant adipocyte differentiation), high $[Ca^{2+}]_o$ in BMSCs enhances the accumulation of adipocytes, while without treatment with insulin and dexamethasone (i.e., a condition of infrequent adipocyte differentiation), high $[Ca^{2+}]_o$ alone does not induce adipocyte accumulation, but rather, enhances osteoblast accumulation.

4. Discussion

In this study, we used primary mouse BMSCs to investigate the effects of high $[Ca^{2+}]_o$ on adipocyte accumulation. High $[Ca^{2+}]_o$ enhanced the accumulation of adipocytes in the presence of treatment with both insulin and dexamethasone, whereas high $[Ca^{2+}]_o$ alone did not induce adipocyte accumulation. This enhanced accumulation was the result of both enhanced proliferation of BMSCs and their differentiation into adipocytes. In addition, high $[Ca^{2+}]_o$ induced an increase in $[Ca^{2+}]_i$ in BMSCs, and an intracellular Ca^{2+} chelator suppressed the enhancement in adipocyte accumulation resulting from high $[Ca^{2+}]_o$. These data indicate a new role for extracellular Ca^{2+}: increased $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ enhance the accumulation of adipocytes in the bone marrow stroma.

It has been reported that increased $[Ca^{2+}]_i$ inhibits adipocyte differentiation from the 3T3-L1 cells derived from mouse embryo fibroblasts [13–15]. B. Jensen et al. have reported that high $[Ca^{2+}]_o$ inhibits the differentiation of adipocytes from 3T3-L1 cells without affecting $[Ca^{2+}]_i$ [16]. However, in this study, we show that high $[Ca^{2+}]_o$ induces an increase in $[Ca^{2+}]_i$, which enhances the differentiation of adipocytes from BMSCs (Fig. 3). Although the reason for this discrepancy is not clear, a possible explanation is the difference in cell models. Hang Shi et al. [17] have reported that increased $[Ca^{2+}]_i$ shows a stimulatory effect in the late differentiation stages of pre-adipocytes derived from human stromal-vascular cells in subcutaneous fat deposits, which differs from the report that increased $[Ca^{2+}]_i$ does not show effects during the late stages of 3T3-L1 cell differentiation [13]. Furthermore, we show that IBMX, the important inducer of adipocyte differentiation from 3T3-L1 cells, did not affect adipocyte differentiation from BMSCs (Fig. 1A). Therefore, cell models derived from different tissues and developmental stages may exhibit distinct differentiation properties.

The bone marrow stroma contains both osteoblasts and adipocytes that have a common precursor, the pluripotent mesenchymal stem cell found in BMSCs [1–3]. While in young and normal physiological states, osteoblasts predominate, adipocytes gradually increase with aging and diseases that include osteoporosis and diabetes [18–21]. Recent studies have shown that adipocytes in the bone marrow suppress lymphohematopoesis [22,23]. Thus, antagonizing marrow adipocyte development may be a novel

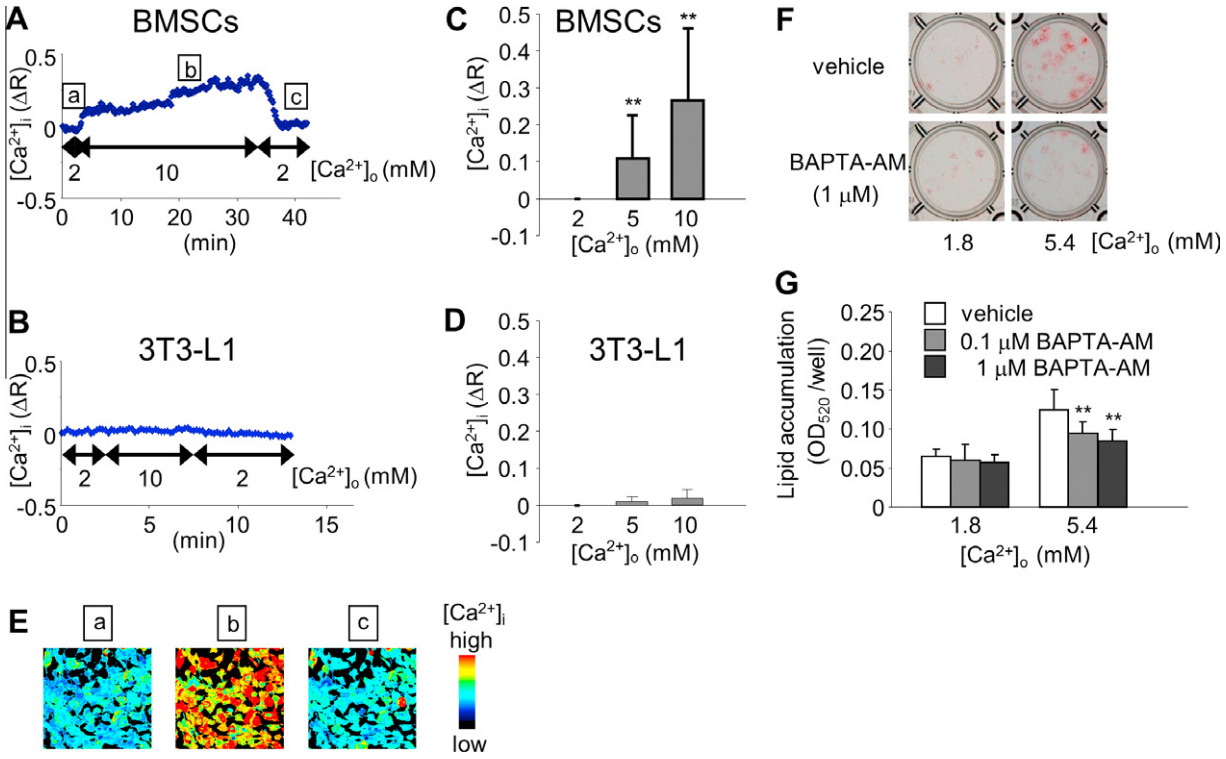

Fig. 3. An intracellular Ca^{2+} chelator suppresses the enhancement of adipocyte accumulation. The levels of cellular $[Ca^{2+}]_i$ were measured using the fura-2 method. Typical records showing the time course of $[Ca^{2+}]_i$ in BMSCs (A) and 3T3-L1 cells (B). Changes in $[Ca^{2+}]_i$ levels caused by changes in $[Ca^{2+}]_o$ in BMSCs (C) and 3T3-L1 cells (D). (E) The pictures show the pseudocolor images in fura-2-AM-loaded BMSCs, where red and yellow represent high and blue and black represents low $[Ca^{2+}]_i$, were acquired at the times corresponding to a-c in panel (A). n > 26 (** P < 0.01 vs. control). (F, G) BMSCs were cultured in differentiation medium with the indicated $[Ca^{2+}]_o$ under the treatment of BAPTA-AM, an intracellular calcium chelator. (F) Typical photomicrographs of Oil Red O staining of the cells are shown. (G) The level of adipocyte accumulation was measured by Oil Red O extraction. n = 6 (* P < 0.05, ** P < 0.01 vs. 5.4 mM of $[Ca^{2+}]_o$ group treated with vehicle).

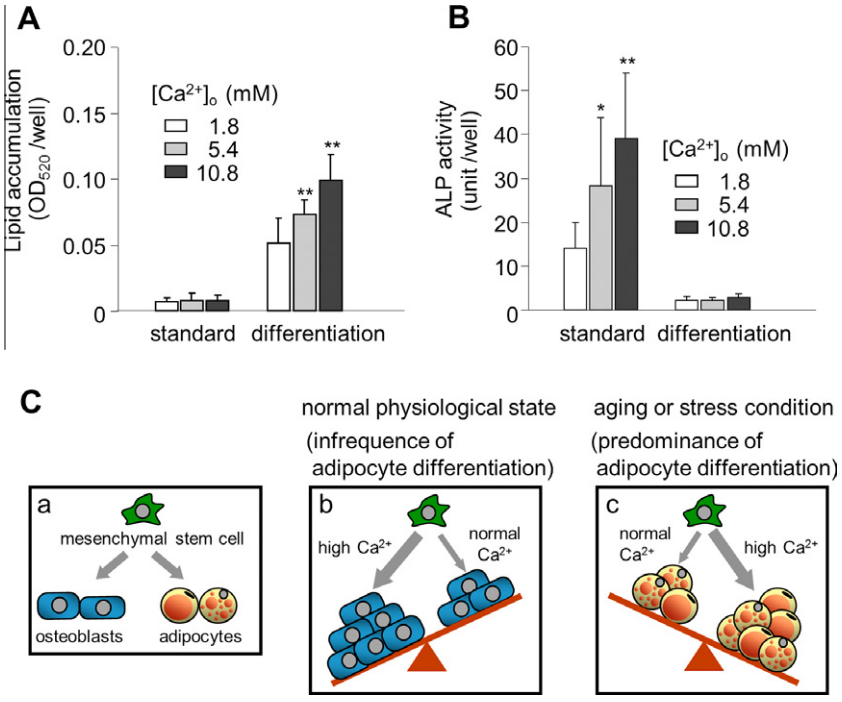

Fig. 4. Effects of high $[Ca^{2+}]_o$ on bone marrow stromal cells differ based on culture conditions. BMSCs were cultured in standard or differentiation medium with the indicated $[Ca^{2+}]_o$. After 14 days, the levels of both adipocyte and osteoblast accumulation were measured by Oil Red O extraction (A) and an alkaline phosphatase (ALP) activity assay (B), respectively. n = 6–10 (*$P < 0.05$, **$P < 0.01$ vs. 1.8 mM of $[Ca^{2+}]_o$ group). (C) Possible roles of bone marrow Ca^{2+} on the bone marrow stroma. (a) Bone marrow mesenchymal stem cells have the potential to differentiate into osteoblasts and adipocytes, and this balance of differentiation direction decides the bone marrow environment. Mesenchymal stem cells differentiate into osteoblasts under normal physiological conditions, but differentiate into adipocytes under aging or stress conditions. We propose that increased $[Ca^{2+}]_o$ enhances osteoblast development to form bone under conditions of infrequent adipocyte differentiation (b) and adipocyte accumulation under conditions of predominant adipocyte differentiation (c).

approach for treating diseases caused by decreased lymphohematopoiesis such as age-related anemia. Additionally, because high levels of marrow adipocytes are a risk factor for fractures [24–27], the suppression of marrow adipocyte accumulation may provide a new preventive approach for fractures caused by aging and diseases. We show that an intracellular Ca^{2+} chelator suppressed the enhancement in adipocyte accumulation caused by high $[Ca^{2+}]_o$ (Fig. 3F, G). Thus, intracellular Ca^{2+} signaling may be a new target for therapy for anemia and fractures caused by accelerated marrow adipocyte accumulation.

Local bone marrow Ca^{2+} levels can reach high concentrations due to bone resorption [4], which is one of the notable features of the bone marrow stroma. Both extracellular and intracellular Ca^{2+} are versatile signaling molecules that are involved in the regulation of cell functions including proliferation, differentiation, and cell death. Bone homeostasis is maintained by both the balance between osteoblastic bone formation and osteoclastic bone resorption [28] and the balance of mesenchymal stem cell differentiation into osteoblasts and adipocytes. Bone resorption by osteoclasts, which are derived from hematopoietic stem cells, gives rise to a release of Ca^{2+}. Increased bone marrow Ca^{2+} enhances the chemotaxis [5,6], proliferation [5,7], and differentiaztion [8,9] of osteoblasts to reform missing bone during normal physiological conditions. However, despite the increased extracellular Ca^{2+} resulting from bone resorption, bone mass declines and adipocyte levels in the marrow increase as a result of aging and some diseases such as diabetes and osteoporosis. This has been explained by imbalances in which osteoclastic bone resorption predominates over osteoblastic bone formation and adipocyte differentiation predominates over osteoblast differentiation. Under conditions in which adipocyte differentiation predominates, however, the effects of increased extracellular Ca^{2+} on the bone marrow, including whether high Ca^{2+} helps to reform bone or affects adipocyte accumulation, remain largely unknown.

We observed that without insulin and dexamethasone treatment (i.e., a condition of infrequent adipocyte differentiation), high $[Ca^{2+}]_o$ enhanced osteoblast but not adipocyte accumulation in BMSCs (Fig. 4A, B). However, it is important to note that treatment with insulin and dexamethasone (i.e., a condition of predominant adipocyte differentiation), high $[Ca^{2+}]_o$ enhanced adipocyte but not osteoblast accumulation in BMSCs (Fig. 4A, B). We propose that increased bone marrow $[Ca^{2+}]_o$ enhances osteoblast development to form bone under conditions of infrequent adipocyte differentiation such as the normal physiological state, and enhances adipocyte accumulation under conditions of predominant adipocyte differentiation such as aging and stress (Fig. 4C). Our hypothesis might explain a part of the reason why bone mass declines and adipocyte levels in the marrow increase in people during aging and some diseases such as diabetes and osteoporosis despite increased extracellular Ca^{2+} caused by bone resorption; it is known that adipocyte differentiation predominates over osteoblast differentiation in these conditions. Therefore, the increased $[Ca^{2+}]_o$ caused by bone resorption might accelerate adipocyte accumulation instead of osteoblastic bone formation in aging, diabetic and osteoporotic patients.

In summary, we show that high $[Ca^{2+}]_o$ enhances osteoblast accumulation in BMSCs under conditions of infrequent adipocyte differentiation, while under conditions of predominant adipocyte differentiation, high $[Ca^{2+}]_o$ enhances adipocyte accumulation through an increase in $[Ca^{2+}]_i$ levels. Thus, increases in $[Ca^{2+}]_o$ caused by bone resorption might accelerate adipocyte accumulation instead of osteoblastic bone formation in aging, diabetic and osteoporotic patients, in which adipocyte differentiation has become dominant. In addition, controlling extracellular and intracellular Ca^{2+} concentrations may provide a new therapeutic approach that can govern the balance of adipocyte and osteoblast development.

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