The Journal of Physiological Sciences (2019) 69:741–748
https://doi.org/10.1007/s12576-019-00690-9

ORIGINAL PAPER

Phorbol 12-myristate 13-acetate (PMA) suppresses high Ca^{2+}-enhanced adipogenesis in bone marrow stromal cells

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Received: 4 March 2019 / Accepted: 18 June 2019 / Published online: 29 June 2019
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Abstract

We have previously reported that increased extracellular and intracellular Ca^{2+} lead to adipocyte accumulation in bone marrow stromal cells (BMSCs). However, strategies to suppress high Ca^{2+}-enhanced adipocyte accumulation have not been reported. We examined the effects of the diacylglycerol analog phorbol 12-myristate 13-acetate (PMA) on proliferation and adipogenesis of mouse primary BMSCs. We used 9 mM $CaCl_2$ and 100 nM ionomycin to increase extracellular Ca^{2+} and intracellular Ca^{2+}, respectively. PMA suppressed the expression of both C/EBPα and PPARγ under normal adipogenesis, adipogenesis + $CaCl_2$, and adipogenesis + ionomycin conditions. PMA enhanced proliferation under normal adipogenesis conditions but suppressed proliferation under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions. PMA did not affect the accumulation of adipocytes under normal adipogenesis conditions but suppressed adipocyte accumulation under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions. These results suggest that the PMA-dependent pathway is an important signaling pathway to suppress high Ca^{2+}-enhanced adipocyte accumulation.

Keywords Bone marrow stromal cells · Adipocytes · Extracellular Ca^{2+} · Intracellular Ca^{2+} · Diacylglycerol · PMA

Introduction

Adipocytes in bone marrow gradually predominate during aging [1] and obesity [1], the use of glucocorticoids [2, 3], and postmenopause [4, 5]. Since a high level of marrow adipocytes is a risk factor for suppressed lymphohematopoiesis [6, 7] and fractures [8–11], suppression of marrow adipocyte accumulation may provide a new preventive approach for decreased lymphohematopoiesis and fractures caused by aging and diseases. Local bone marrow Ca^{2+} levels can reach high concentrations of 40 mM by bone resorption [12], which is a notable peculiarity of the bone marrow stroma. We have previously reported that increased extracellular and intracellular Ca^{2+} lead to adipocyte accumulation rather than osteoblastic bone formation in aging and diabetes mellitus patients [13, 14]. However, strategies to suppress high Ca^{2+}-enhanced adipocyte accumulation have not been reported.

Diacylglycerol is a versatile signaling molecule that is involved in the regulation of many cell functions, including proliferation, differentiation, and cell death. It has recently been reported that the diacylglycerol analog phorbol 12-myristate 13-acetate (PMA) may enhance proliferation and suppress adipogenesis [15]. However, the effect of PMA on high Ca^{2+}-enhanced adipocyte accumulation has not been elucidated. In the present study, we examined the effects of PMA on the proliferation and adipogenesis of BMSCs under both high extracellular and intracellular Ca^{2+} conditions. To mimic the bone marrow niche in vivo, we used crude BMSCs (many types of bone marrow adherent cells, including mesenchymal stem cells, osteoblasts, chondrocytes, and adipocytes) [16–18]. In this study, we show that PMA suppresses high Ca^{2+}-enhanced adipocyte accumulation through suppression of proliferation of BMSCs and differentiation into adipocytes.

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Materials and methods

Cell culture

The cell culture methods were described previously [19, 20]. Briefly, male C57Bl/6 mice (Charles River Japan, Kanagawa, Japan) were euthanized by cervical dislocation, and bone marrow cells were collected from the femur and tibia and cultured at 37 °C in 5% CO_2/95% air. We selectively maintained adherent cells (BMSCs) by removing floating cells when changing the medium. This 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.

Quantitative real-time RT-PCR analysis

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). 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), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assay identification number Mm99999915_g1) were used. PCR mixtures were preincubated at 50 °C for 2 min, followed by 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. Real-time data were analyzed using 7500 software (Applied Biosystems).

Measurement of cell numbers, cell viability, and adipocyte accumulation

To induce adipocyte differentiation, cells were seeded onto 24-well plates at 3×10^4 cells per well and treated with 10 µg/ml insulin and 0.25 µM dexamethasone for 14 days. Cell counts were estimated using both a hematocytometer and a metabolic assay using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5- (2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8) assay [21], which is a modification of the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, 500 µl of medium and 50 µl of WST-8 reagent (Dojindo, Kumamoto, Japan) were added to the wells. After a 4 h incubation at 37 °C, absorbance at 450 nm was recorded with a microtiter plate reader. To evaluate cell viability, cells were incubated with 100 ng/ml propidium iodide (PI) for 1 min in the dark, and the ratio of non-stained cells was evaluated with a FACScan flow cytometer (Becton–Dickinson, San Jose, CA, USA). We used unstained cells as a negative control and fixed cells with 4% formaldehyde for 15 min as a positive control for PI staining. For Oil Red O staining and extraction, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and rinsed twice with PBS. The cells were washed with 60% isopropanol and then treated with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 60% isopropanol for 20 min. The cells were rinsed three times with 60% isopropanol. Pictures of the cells were taken, and Oil Red O dye in lipid droplets was eluted into 500 µl of isopropanol. Absorbance at 520 nm was finally measured with a microtiter plate reader.

Immunoblotting

Cells were treated with 100 nM phorbol-myristate-acetate (PMA) for 10 min and overnight. After treatment, the cells were washed with PBS two times and lysed in 100 µl of RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein concentration of the cell lysate was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific) and standardized with bovine serum albumin. The cell extract was diluted (1:1) in 2 × sample buffer (100 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 12% β-mercaptoethanol, 0.04% bromophenol blue) and then boiled for 5 min at 95 °C. Equal amounts of proteins were loaded onto an SDS-10% polyacrylamide gel and resolved by one-dimensional SDS-PAGE at a constant current of 20 mA/gel. After the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, and 15% methanol), the proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore Corporation, Darmstadt, Germany) at 100 V for 60 min on ice (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, Inc., Hercules CA, USA). The membrane was blocked with PVDF blocking reagent (Toyobo Co., Ltd., Osaka, Japan) and incubated with appropriate dilutions of the primary antibody in Can Get Signal Solution 1 (NKB-201 Toyobo Co.) at 4 °C overnight. The following primary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA): phospho-ERK1/2 (Thr202/Tyr204, 1:1000) and ERK1/2 (1:2000). After the membrane was washed with TBS-T (Tris-buffered saline and 0.1% Tween-20), it was incubated for 1 h at room temperature with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling) in secondary antibody

solution 2 (NKB-301Toyobo Co.). Blots were detected using ECL Prime reagent (GE Healthcare, Waukesha, WI, USA), and signals were obtained with the Luminescent Image Analyzer (Fuji Photo Film, Tokyo, Japan). Analyses were performed using ImageJ software (version 1.47v, National Institutes of Health, MD, USA).

Statistical analysis

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

Results

PMA suppresses adipocyte differentiation and enhances the proliferation of BMSCs under adipogenesis conditions

We first examined the effects of PMA under adipogenesis conditions. PMA (100 nM) suppressed the expression of both C/EBPα and PPARγ (Fig. 1a) and enhanced the proliferation of BMSCs (Fig. 1b). PMA (100 nM) did not affect the accumulation of adipocytes (Fig. 1c). These results indicate that PMA enhances the proliferation of BMSCs and suppresses adipocyte differentiation, which offset each other, resulting in a small change of the accumulation of adipocytes.

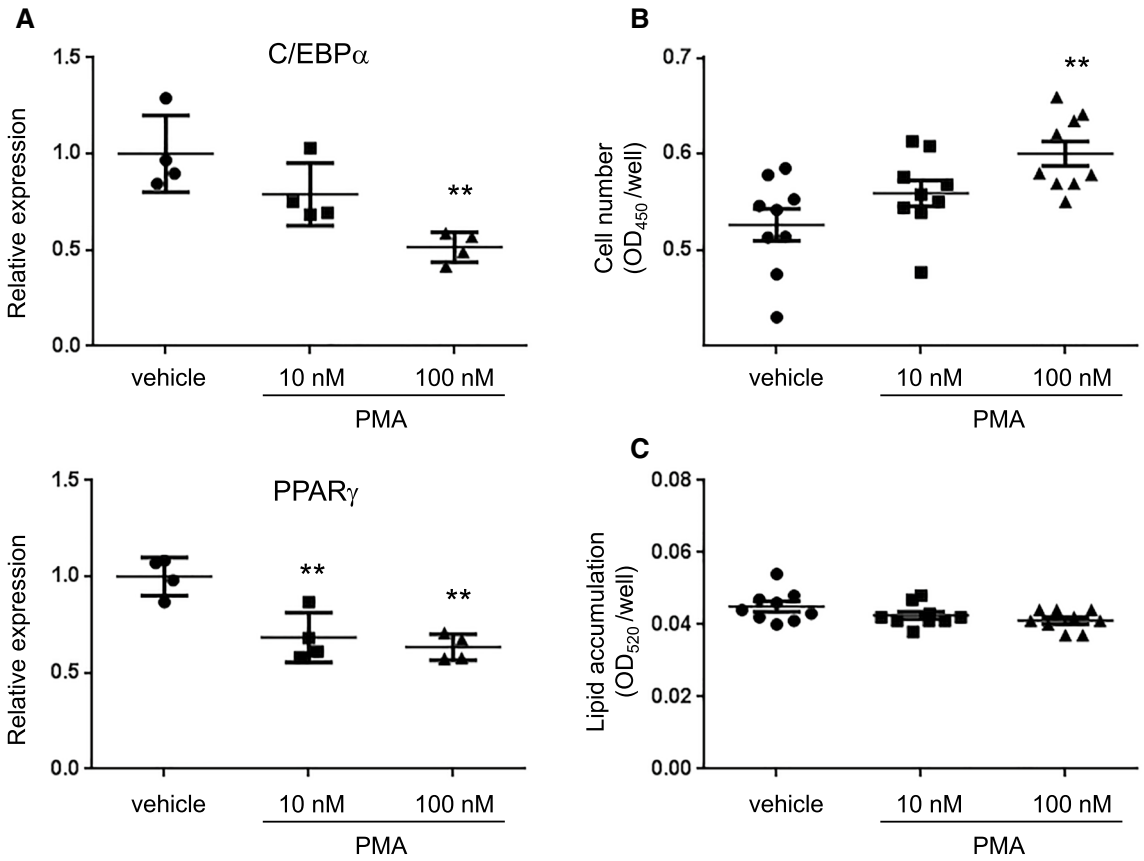

Fig. 1 PMA suppresses adipocyte differentiation and enhances the proliferation of BMSCs under adipogenesis conditions. **a** Bone marrow stromal cells (BMSCs) were cultured in standard medium. After 7 days, cells were cultured for 1 day in adipocyte differentiation medium and treated with phorbol 12-myristate 13-acetate (PMA) at the indicated concentration. Total RNA was isolated, and the quantitative mRNA levels of CCAAT-enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) were determined using real-time quantitative RT-PCR. $n = 4$ ($^{**}P < 0.01$ vs. vehicle control). **b, c** BMSCs were cultured in adipocyte differentiation medium for 14 days with the indicated concentration of PMA. **b** The cell numbers were evaluated using a modification of the MTT assay. $n = 9$ ($^{**}P < 0.01$ vs. vehicle control). **c** The levels of total adipocyte accumulation were measured based on Oil Red O extraction. $n = 9$ (not significant)

PMA activates the MAPK/ERK pathway

PMA is a direct protein kinase C (PKC) activator. It is known that activation of PKC can directly phosphorylate Raf-1 [22, 23], which in turn, sequentially activates and phosphorylates extracellular signal-regulated kinase (ERK). We next determined whether PMA affects the activity of ERK in BMSCs using Western blotting. ERK phosphorylation was increased after 10 min and declined after 100 nM PMA treatment overnight (Fig. 2a, b).

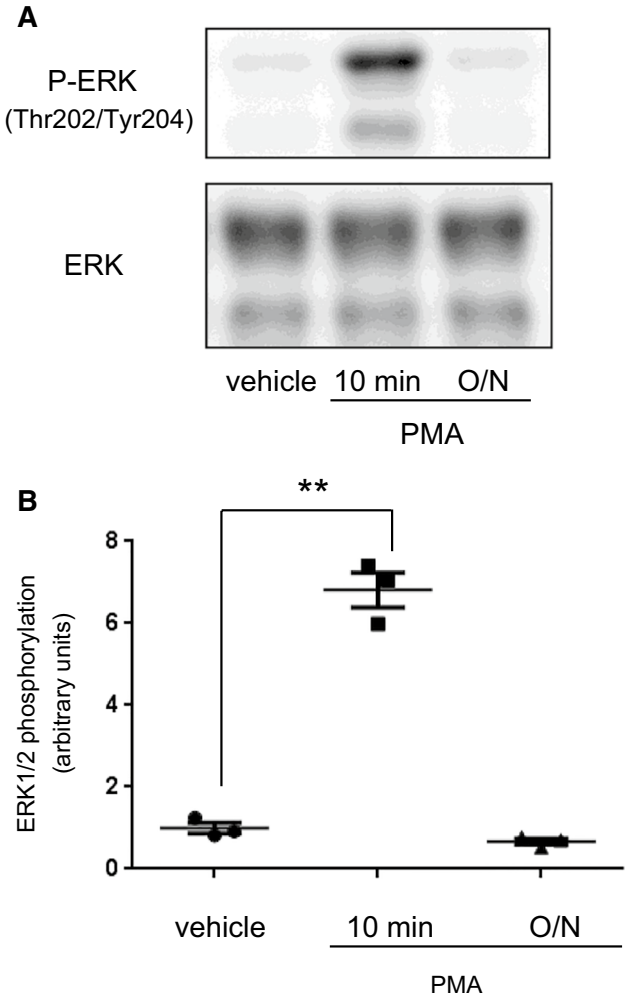

Fig. 2 PMA activates the MAPK/ERK pathway. BMSCs were cultured in standard medium for 7 days. Cells were lysed at different time points (pretreatment and 10 min and overnight (O/N) after 100 nM PMA treatment), and the cell extracts (protein) were analyzed by Western blotting. **a** Representative blots of ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) are shown. **b** The *bar graph* shows the densitometric analysis of phosphorylation normalized to the basal expression of ERK1/2. $n = 3$ ($^{**}P < 0.01$ vs. vehicle control)

Inhibition of ERK increases adipocyte differentiation and decreases the proliferation of BMSCs under adipogenesis conditions

We next assessed the effects of ERK inhibition on both adipogenesis and the proliferation of BMSCs using mitogen-activated protein kinase/ERK kinase (MEK) inhibitors. Treatment with the inhibitors 5 μM U0126 and 20 nM PD0325901 increased the expression of both C/EBPα and PPARγ (Fig. 3a). On the other hand, treatment with 5 μM U0126 and 20 nM PD0325901 suppressed the proliferation of BMSCs (Fig. 3b). These results indicate that PMA enhances the proliferation of BMSCs and suppresses adipocyte differentiation through phosphorylation of ERK.

PMA suppresses both adipocyte differentiation and the proliferation of BMSCs under both adipogenesis and high extracellular Ca^{2+} conditions

We have reported that high extracellular Ca^{2+} enhances both the proliferation of BMSCs and their differentiation into adipocytes, resulting in enhanced adipocyte accumulation [13, 14]. We next examined the effects of PMA under adipogenesis conditions with increased extracellular Ca^{2+}. Treatment with $CaCl_2$ (+9 mM) increased the expression of both C/EBPα and PPARγ (Fig. 4a). The addition of 100 nM PMA to $CaCl_2$ (+9 mM) suppressed the enhanced expression of both C/EBPα and PPARγ (Fig. 4a). Treatment with $CaCl_2$ (+9 mM) enhanced the proliferation of BMSCs (Fig. 4b). This enhanced proliferation of BMSCs was suppressed by the addition of 100 nM PMA to $CaCl_2$ (+9 mM, Fig. 4b). However, cell viability was not different among the control, 100 nM PMA, $CaCl_2$ (+9 mM), and $CaCl_2$ (+9 mM) + 100 nM PMA (Fig. 4c) groups. Treatment with $CaCl_2$ (+9 mM) enhanced the accumulation of adipocytes, which was suppressed by the addition of 100 nM PMA to $CaCl_2$ (+9 mM, Fig. 4d).

PMA suppresses both adipocyte differentiation and the proliferation of BMSCs under both adipogenesis and high intracellular Ca^{2+} conditions

We have reported that increased intracellular Ca^{2+} enhances the proliferation of BMSCs without affecting their differentiation into adipocytes, resulting in enhanced adipocyte accumulation [13, 14]. We next examined the effects of PMA under adipogenesis conditions with increased intracellular Ca^{2+}. We used a Ca^{2+} ionophore, ionomycin, to increase intracellular Ca^{2+}. Treatment with 100 nM ionomycin did not affect the expression of either C/EBPα or PPARγ (Fig. 4a). The addition of 100 nM PMA to 100 nM

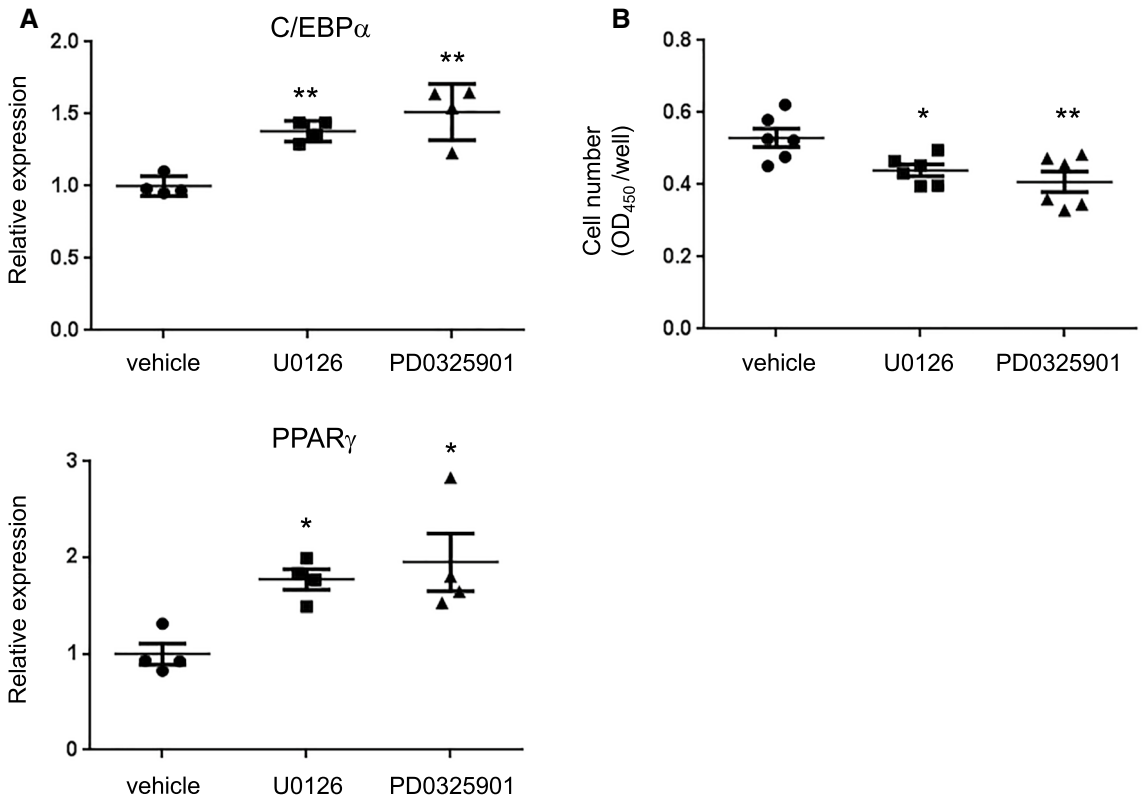

Fig. 3 Inhibition of ERK increases adipocyte differentiation and decreases the proliferation of BMSCs under adipogenesis conditions. **a** BMSCs were cultured in standard medium. After 7 days, the cells were cultured for 1 day in adipocyte differentiation medium and treated with mitogen-activated protein kinase/ERK kinase (MEK) inhibitors (5 μM U0126 or 20 nM PD0325901). Total RNA was isolated, and the quantitative mRNA levels of C/EBPα and PPARγ were determined using real-time quantitative RT-PCR. $n=4$ ($^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle control). **b** BMSCs were cultured in adipocyte differentiation medium for 14 days with 5 μM U0126 or 20 nM PD0325901. The cell numbers were evaluated using a modification of the MTT assay. $n=6$ ($^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle control)

ionomycin suppressed the expression of both C/EBPα and PPARγ (Fig. 4a). Treatment with 100 nM ionomycin enhanced the proliferation of BMSCs (Fig. 4b). This enhanced proliferation of BMSCs was suppressed by the addition of 100 nM PMA to 100 nM ionomycin (Fig. 4b). However, cell viability was not different among the control, 100 nM PMA, 100 nM ionomycin, and 100 nM ionomycin + 100 nM PMA groups (Fig. 4c). Treatment with 100 nM ionomycin enhanced the accumulation of adipocytes, which was suppressed by the addition of 100 nM PMA to 100 nM ionomycin (Fig. 4d).

Discussion

In the present study, we examined the effects of the diacylglycerol analog PMA on the proliferation and adipogenesis of mouse primary BMSCs. We showed that PMA suppressed the expression of both C/EBPα and PPARγ under normal adipogenesis, adipogenesis + $CaCl_2$, and adipogenesis + ionomycin conditions. PMA enhanced the proliferation of BMSCs under normal adipogenesis but suppressed the proliferation of BMSCs under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions. We also showed that PMA did not affect the accumulation of adipocytes under normal adipogenesis conditions but suppressed the accumulation of adipocytes under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions. These results suggest that the PMA-dependent pathway is an important signaling pathway to suppress high Ca^{2+}-enhanced adipocyte accumulation.

The antiadipogenic effect of PMA has been reported in human adipose tissue-derived stromal cells [15]. In the present study, we showed that PMA in mouse BMSCs suppressed the expression of both C/EBPα and PPARγ, which are important adipogenic transcription factors (Fig. 1a). This antiadipogenic effect was also observed under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions (Fig. 4a). It is well known that PMA directly activates PKC and that activated PKC can directly phosphorylate Raf-1 [22, 23], which in turn, sequentially activates and phosphorylates ERK. We showed that PMA increased the phosphorylation of ERK (Fig. 2) and that ERK inhibition increased the

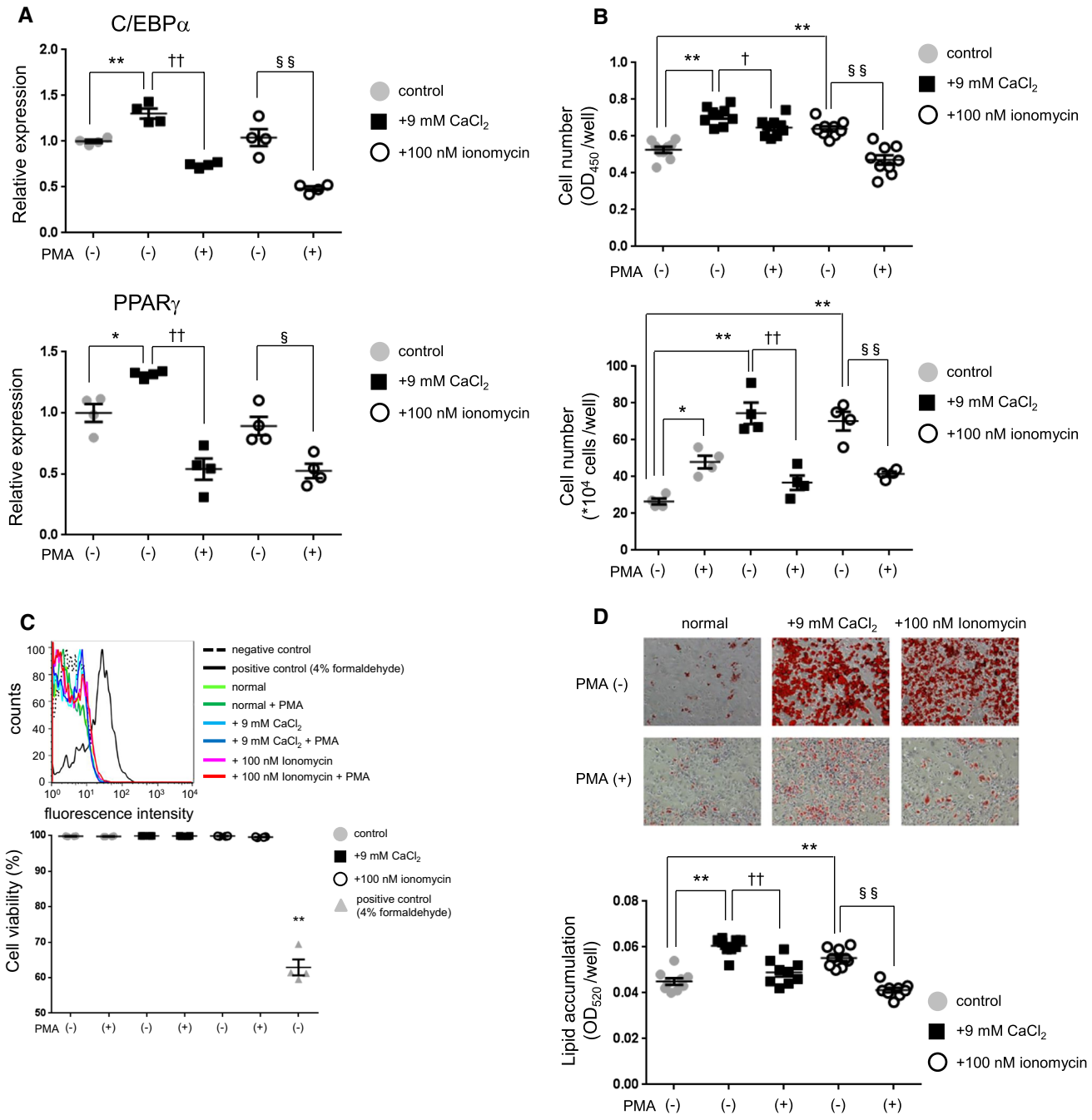

Fig. 4 PMA suppresses both adipocyte differentiation and the proliferation of BMSCs under both adipogenesis and high-Ca^{2+} conditions. **a** BMSCs were cultured in standard medium. After 7 days, the cells were cultured for 1 day in adipocyte differentiation medium with addition of 9 mM $CaCl_2$ or 100 nM ionomycin and/or 100 nM PMA. Total RNA was isolated, and the mRNA levels of C/EBPα and PPARγ were determined using real-time quantitative RT-PCR ($n=4$). **b–d** BMSCs were cultured in adipocyte differentiation medium for 14 days with addition of 9 mM $CaCl_2$ or 100 nM ionomycin and/or 100 nM PMA. (**b**-*top*) The cell numbers were evaluated using a modification of the MTT assay ($n=9$). (**b**-*bottom*) The cell numbers were counted using a hematocytometer ($n=4$). **c** Cell viability was measured by propidium iodide (PI) uptake and flow cytometry. A representative histogram of cells is shown in the *top panel*, and the cell viability is shown in the *bottom panel* ($n=4$). **d** Typical photomicrographs of Oil Red O staining of cells are shown in the *upper panels*. *Bars* indicate 100 μm. The levels of total adipocyte accumulation were measured based on Oil Red O extraction and shown in the *bottom panel* ($n=9$). **a–d** $^{*}P<0.05$, $^{**}P<0.01$ vs. control without PMA, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. +9 mM $CaCl_2$ without PMA, $^{\S}P<0.05$, $^{\S\S}P<0.01$ vs. +100 nM ionomycin without PMA

expression of both C/EBPα and PPARγ (Fig. 3a). Our data are supported by some reports showing that ERK activation inhibits adipogenesis [24, 25]. These results suggest that PMA suppresses adipocyte differentiation through ERK phosphorylation. Transient ERK activation is typical of proliferation, whereas sustained ERK activation is characteristic of differentiation [26–28]. In the present study, we showed that PMA affected both the differentiation and proliferation of BMSCs (PMA suppressed the adipocyte differentiation and enhanced the proliferation of BMSCs) under normal adipogenesis (Fig. 1a, b), although increased phosphorylation of ERK was not sustained (Fig. 2). In adipogenesis of some cell types, transient phosphorylation of ERK might inhibit adipocyte differentiation because transient depletion of ERK1/2-enhanced adipocyte differentiation has been reported [29].

We have previously reported that high $CaCl_2$ might activate Ca^{2+}-sensing receptor (CaSR), which increases the intracellular Ca^{2+}, decreases cAMP concentration, and suppresses the phosphorylation of ERK in BMSCs [13, 20]. We also reported that ionomycin increases intracellular Ca^{2+} but does not affect cAMP concentration or ERK phosphorylation in BMSCs. In these previous reports, we suggested that decreased cAMP and suppressed ERK phosphorylation enhance adipogenesis and that increased intracellular Ca^{2+} enhances proliferation. In the present study, the proliferation of BMSCs was promoted by high Ca^{2+} (+9 mM $CaCl_2$ or +100 nM ionomycin) or PMA. However, the combination of high Ca^{2+} and PMA suppressed cell proliferation. To our knowledge, this phenomenon has not been previously reported in BMSCs or other cell types. A possible explanation for this discrepancy is the difference in the differentiation state of BMSCs because the effects of intracellular Ca^{2+} on cell proliferation are dependent on cell types [30–33], and we observed that PMA suppresses the adipogenesis of BMSCs. In other words, high Ca^{2+} might enhance the proliferation of BMSCs committed to adipocytes and have little effect on the proliferation of uncommitted BMSCs.

In the accumulation of bone marrow adipocytes, two important key factors are adipocyte differentiation and the proliferation of BMSCs, which have the potential to differentiate into adipocytes. Under normal adipogenesis conditions, PMA enhanced the proliferation of BMSCs (Fig. 1b) and suppressed adipocyte differentiation (Fig. 1a), which offset each other, resulting in a small change of the accumulation of adipocytes (Fig. 1c). On the other hand, under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions (Fig. 4d), PMA suppressed the accumulation of adipocytes. This suppression of accumulation by PMA was caused by both suppressed adipocyte differentiation (Fig. 4a) and suppressed proliferation of BMSCs (Fig. 4b). Although further evaluation is needed to interpret the inverse effects on the proliferation of BMSCs, it is of interest that the PMA-dependent pathway is an important signaling pathway to suppress enhanced adipocyte accumulation under increased extracellular and intracellular Ca^{2+} conditions. Adipocytes in bone marrow gradually increase during aging [1] and obesity [1], the use of glucocorticoids [2, 3], and postmenopause [4, 5]. Many studies have shown that the accumulation of adipocytes in the bone marrow suppresses lymphohematopoiesis [6, 7] and is a risk factor for fractures [8–11]. Thus, reagents that possibly increase diacylglycerol may be new targets of therapy for anemia and fractures caused by accelerated marrow adipocyte accumulation.

In summary, under adipogenesis + $CaCl_2$ and adipogenesis + ionomycin conditions, PMA suppressed the accumulation of adipocytes, while under normal adipogenesis conditions, PMA had little effect on the accumulation of adipocytes. These results suggest that the PMA-dependent pathway is an important signaling pathway to suppress enhanced adipocyte accumulation under increased extracellular and intracellular Ca^{2+} conditions. Because the accumulation of adipocytes in the bone marrow is a risk factor for anemia and fractures, reagents that possibly increase diacylglycerol may be new targets of therapy for anemia and fractures caused by accelerated marrow adipocyte accumulation.

Funding This work was supported by JSPS KAKENHI grant numbers JP18K17977 (to R.H.) and JP16K01836 (to Y.K.).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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