Osteogenic gene expression induced by mineral trioxide aggregate via calcium-sensing receptor in murine MC3T3-E1 cells

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Contents

This thesis is composed by an article ahead of print and an additional data listed below.

1. Yasukawa T, Hayashi M, Tanabe N, Tsuda H, Suzuki Y, Kawato T, Suzuki N, Maeno M, Ogiso B. Involvement of the calcium-sensing receptor in mineral trioxide aggregate-induced osteogenic gene expression in murine MC3T3-E1 cells. Dental Materials Journal, in press.

2. Alkaline phosphatase mRNA expression data suggesting the high biocompatibility of mineral trioxide aggregate.

Abstract

Mineral trioxide aggregate (MTA) has excellent biocompatibility as well as bioactivity, including the induction of osteoblast differentiation. Thus, MTA has been widely used in endodontic treatments, such as direct pulp capping, root-end filling, perforation repair, and apexification. MTA contains primarily calcium silicate, which is converted into calcium hydroxide upon contact with tissue fluid. In turn, the calcium hydroxide dissociates into calcium and hydroxide ions, which disperse into the surrounding environment, resulting in MTA bioactivity. Given these findings, the release of calcium ions from MTA may be an important characteristic underlying the biological effect of MTA.

The calcium-sensing receptor (CaSR), a member of the G protein-coupled receptor superfamily is a key mediator of the direct actions of calcium ions on the parathyroid, and it regulates homeostatic responses that restore calcium ions in the blood to normal level. Also, osteoblasts have been shown to express CaSR, and local changes in the concentrations of extracellular calcium ions can be sensed by CaSR, resulting in regulation of osteoblastic proliferation, differentiation, and mineralization. Thus, CaSR in osteoblasts is thought to be important for bone metabolism. Here, it was hypothesized that calcium ions released from MTA might directly induce the levels of osteogenic gene expression via CaSR. The purpose

of this study was to examine the role of CaSR during the early stage of MTA-induced differentiation.

ProRoot MTA and mouse calvarial cell line MC3T3-E1 cells were used for the experiments. MC3T3-E1 cells were cultured with or without (control) MTA for 3 days using cell culture insert. The effect of MTA on cell proliferation and alkaline phosphatase (ALP) gene expression was examined to assess the biocompatibility of MTA by Cell Counting Kit 8 and real-time polymerase chain reaction (PCR) analysis, respectively. Cell numbers and ALP mRNA expressions in both MTA and control groups increased in a time-dependent manner. The significant higher cell number and expression were observed in MTA group on day 3, compared to that of control. Next, the expressions of Runx2, type I collagen, and CaSR were analyzed by PCR and enzyme-linked immunosorbent assay analyses in mRNA and protein levels, respectively. These levels were increased significantly in cells exposed to MTA compared with control. Then, calcium ions released from MTA were analyzed on days 1, 2, and 3 after the placement of MTA. The concentrations of calcium ions released from MTA increased in a time-dependent manner, and reached ~2.5 mM on day 3. Finally, MC3T3-E1 cells were cultured with MTA and EGTA (a calcium chelator) or NPS2143 (a CaSR antagonist). The expression levels of Runx2, type I collagen, and CaSR mRNAs were decreased to control levels by MTA plus EGTA or NPS2143.

The present results suggest the calcium ions released from MTA promoted the expression of osteogenic and CaSR genes via CaSR.

Introduction

Mineral trioxide aggregate (MTA) shows excellent biocompatibility as well as bioactivity in dental pulp and periodontal tissues. Thus, MTA has been widely used in endodontic treatments, including direct pulp capping, root-end filling, perforation repair, and apexification (1-3). MTA bioactivity involves the ability to enhance cell proliferation, migration, and mineralization in various cell types (4-9). MTA contains primarily calcium silicate, which is converted into calcium hydroxide upon contact with tissue fluid. In turn, the calcium hydroxide dissociates into calcium and hydroxide ions, which disperse into the surrounding environment, resulting in MTA bioactivity (7).

Some *in vitro* studies have indicated that calcium ions are released continuously from MTA (10-12). Takita et al. reported that the release of ions from MTA provides the optimum amount of calcium for the proliferation and migration of dental pulp cells (4,13). In another study involving undifferentiated mesenchymal cell cultures, calcium ions from MTA induced differentiation into osteoblast lineages (14). Given these findings, the release of calcium ions from MTA may be an important characteristic underlying the biological effect of MTA.

The calcium-sensing receptor (CaSR), a member of the G protein-coupled receptor superfamily, has been cloned and characterized from the bovine parathyroid gland (15). Many *in vitro* and *in vivo* studies have demonstrated that this receptor is a key mediator of the direct actions of calcium ions on the parathyroid, and it regulates homeostatic responses that restore calcium ions in the blood to normal level (16). Other cell types, including bone and periodontal ligament cells, have been shown to express CaSR (17,18). Especially in osteoblasts, local changes in the concentrations of extracellular calcium ions can be sensed by CaSR, resulting in regulation of their proliferation, differentiation, and mineralization (19). Thus, CaSR in osteoblasts is thought to be important for bone metabolism.

Here, it was hypothesized that calcium ions released from MTA might directly induce the levels of osteogenic gene expression via CaSR. Thus, the purpose of this study was to examine the role of CaSR during the early stage of MTA-induced differentiation.

Materials and Methods

Preparation of the test material and reagents

The test material used was a white MTA powder (ProRoot MTA, Dentsply Tulsa Dental, Johnson City, TN, USA). The MTA powder was mixed according to the manufacturer's protocol, using distilled water as the cement liquid at a liquid/powder ratio of 0.3. Disks of MTA were then prepared using procedures modified from a previously reported method (20). Briefly, the MTA mixture was dispensed into the inverted plastic lids of multiple microcentrifuge tubes. The materials were then placed in a humidified incubator for 24 h at 37°C. The set MTA disks (diameter, 9 mm; thickness, 3 mm) were then removed from the lids and placed in alpha-minimum essential medium (α-MEM, GiBco BRL, Rockville, MD, USA) for 3 days.

Fetal bovine serum (FBS) and other cell culture reagents were obtained from GiBco BRL. All other chemicals were from Sigma Chemicals (St Louis, MO, USA), unless indicated otherwise.

Cell culture

The mouse calvarial cell line MC3T3-E1 (Riken Bio Resource Center, Ibaraki, Japan) was used as a model for osteoblast. Cells were maintained in α-MEM containing 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin solution at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell morphology was observed by phase-contrast microscopy to confirm the maintenance of the culture condition.

Application of the test material in MC3T3-E1 cells

For MTA application, MC3T3-E1 cells were seeded in 6-well culture plates with a culture plate insert containing a porous bottom (3.0-μm pore size, BD Falcon, Franklin Lakes, NJ, USA) at an initial density of ~2×10^4 cells/well in 5 mL of α-MEM. The cells were incubated for 24 h to allow adhesion, and then one MTA disk was placed on each porous bottom (Fig. 1). Cells cultured in the absence of MTA served as the control.

Determination of cell number

The number of cultured cells in the presence or absence of MTA was determined by using the Cell Counting Kit 8 (Dojindo Molecular Technologies Inc, Kumamoto, Japan) on days 1, 2, and 3, as described previously (14). Briefly, the medium was replaced with fresh medium containing 10% (v/v) cell counting reagent at each time point, and the incubation was continued for 2 h. After incubation, the absorbance of the reaction products was measured at 450 nm using a microtiter plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). The cell number was calculated based on the absorbance value relative to standard curve.

Real-time polymerase chain reaction (PCR) analysis

Osteoblastic gene expression levels in the presence or absence of MTA were determined by real-time PCR on days 1, 2, and 3. Total RNA was isolated from the cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA concentrations were measured using the NanoDrop 1000 (ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 0.5 μg of DNase-treated total RNA using the PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan), and the resulting cDNA was analyzed by real-time PCR using the SYBR Green Kit (Takara Bio). Reactions were performed in a total volume of 25 μL containing 12.5 μL SYBR premixed Ex *Taq*, 0.5 μL (20 mM) each primer (Table 1), 9.5 μL dH_2O, and 2 μL (0.5 μg) cDNA. The PCR assays were performed in the Smart Cycler II instrument (Cepheid, Sunnyvale, CA, USA) and analyzed using the Smart Cycler software. The reactions comprised 35 cycles at 95°C for 5 s and 60°C for 20 s. The specificity of the amplified products was verified by melting curve analysis. The calculated values of target gene expression were normalized to that of glyceraldehyde-3-phosphate dehydrogenase used as an internal control.

Enzyme-linked immunosorbent assay (ELISA) analysis

Osteoblastic protein levels in the presence or absence of MTA were determined by ELISA on days 1, 2, and 3. The concentrations of runt-related transcription factor 2 (Runx2) and CaSR in cell lysate and type I collagen in both cell lysate and extracellular matrix around cells were assessed using commercially available sandwich ELISA kits. In brief, phosphate-buffered saline (PBS) was added to culture plate after aspiration of medium. MC3T3-E1 cells were collected with cell scraper and were transferred to a centrifuge tube. The PBS including cells was subjected to ultrasonication for 4 times and was centrifuged to remove cellular debris. The supernatant was directly assayed for ELISA, according to the manufacturer's protocols (Cloud-Clone Corp., Houston, TX, USA). The change in absorbance was measured at 450 nm with a microtiter plate reader. The concentration of each sample was determined by comparing the optical densities of the samples relative to a standard curve.

Measurement of calcium ions released from MTA

For measurement of calcium ion released from MTA in the culture system, the test materials were incubated in α-MEM without cells, with cell culture inserts placed in culture wells. The medium was collected on days 1, 2, and 3 after the placement of MTA. The concentration of calcium ions in the medium was assessed using the Calcium E-Test Kit (Wako Pure Chemical Industries, Osaka, Japan), as described previously (4). Briefly, 1 mL Calcium E-Test reagent and 2 mL buffer were added to 50 μL of the collected medium, and the absorbance of the reaction products was measured at 610 nm using a microtiter plate reader. The concentration of calcium ions was calculated from the absorbance value relative to a standard curve. Also, the calcium ion concentrations were measured in the medium containing 0.3 mM ethylene glycol tetraacetic acid (EGTA), a calcium chelator, or 1.0 μM NPS2143, a CaSR antagonist, in the presence or absence of MTA. Medium without MTA served as the control.

Effect of a calcium chelator on osteoblasticgene expression levels in the presence of MTA

MC3T3-E1 cells were cultured with MTA for 1, 2, and 3 days in α-MEM containing 0.3 mM EGTA, a calcium chelator. Real-time PCR analysis was performed to assess mRNA levels, as described above.

Effect of a CaSR antagonist on osteoblastic gene expression levels in the presence of MTA

The MC3T3-E1 cells were cultured with MTA for 1, 2, and 3 days in α-MEM containing 1.0 μM NPS2143 as a CaSR antagonist. Real-time PCR analysis was performed to assess mRNA levels, as described above.

Effect of a CaSR antagonist on osteoblastic protein level of a CaSR in the presence of MTA

The MC3T3-E1 cells were cultured with MTA for 1, 2, and 3 days in α-MEM containing 1.0 μM NPS2143 as a CaSR antagonist. ELISA analysis was performed to assess protein level of CaSR, as described above.

Statistical analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Student's *t* test and one-way analysis of variance followed by a *post hoc* comparison using Dunnett's test were performed for comparisons with control levels, as appropriate. All statistical assessments were two-sided and evaluated at the 0.05 level of significance.

Results

Effect of MTA on cell culture numbers

The number of cultured cells was examined in the presence or absence of MTA for 1, 2, and 3 days (Fig. 2). MTA increased the cell number and did not inhibit cell proliferation during the study period. The cell number in the presence of MTA was significantly higher than that in the control group on day 3 of culture.

Effect of MTA on alkaline phosphatase gene expression

Levels of alkaline phosphatase (ALP) mRNA in the presence or absence of MTA were assessed by real-time PCR analysis (Fig. 3). MTA increased ALP mRNA and did not affect its expression during the study period. The expression level of ALP in the presence of MTA was significantly higher than that in the control group on day 3 of culture.

Effect of MTA on Runx2, type I collagen, and CaSR gene expression

Levels of Runx2 and type I collagen mRNA in the presence or absence of MTA were assessed by real-time PCR analysis (Fig. 4). Significant upregulations of both mRNAs were observed on days 2 and 3, compared with the control (Fig. 4A, B). The mRNA levels of CaSR, assessed by the same method, significantly increased on day 1 compared with the control group (Fig. 4C).

Effect of MTA on osteoblastic protein production

Levels of osteogenic proteins in the presence or absence of MTA were assessed by ELISA (Fig. 5). In agreement with the mRNA results, levels of Runx2 and type I collagen significantly increased on day 3 compared with the control levels (Fig. 5A, B). Meanwhile, the level of CaSR was significantly increased on days 2 and 3 compared with control levels (Fig. 5C).

Measurement of calcium ions released from MTA

Calcium ions released from MTA disks in the culture system were analyzed after the placement of MTA disk in α-MEM on days 1, 2, and 3 (Fig. 6). The concentrations of calcium ions released from MTA increased in a time-dependent manner, and reached ~2.5 mM on day 3. In contrast, the concentration of calcium ions in the control group was constant, at 1.8 mM throughout the study period. Significant difference was observed between the concentrations in MTA and those in control throughout the study period. Additionally, the calcium ion concentration in the presence of MTA and EGTA was significantly lower than that of control in day 1, however, no significant difference was found in days 2 and 3 (Fig. 6). On the other hand, EGTA alone significantly decreased the calcium ion concentrations during experimental periods (Fig. 6). In contrast, NPS2143 did not affect the calcium ion concentrations (Fig. 6).

Effect of a calcium chelator on osteoblastic gene expression levels in the presence of MTA

MC3T3-E1 cells were cultured in the presence of MTA for 1, 2, and 3 days in α-MEM containing 0.3 mM EGTA, a calcium chelator (Fig. 4). The MTA-induced levels of Runx2, type I collagen, and CaSR decreased to control levels by EGTA. There was no significant difference in the levels of these markers throughout the study period. In addition, no significant difference was observed between treatment with the calcium chelator alone and the control levels.

Effect of a CaSR antagonist on osteoblastic gene expression levels in the presence of MTA

MC3T3-E1 cells were cultured with MTA for 1, 2, and 3 days in α-MEM containing 1.0 μM NPS2143, a CaSR antagonist (Fig. 4). The MTA-induced levels of Runx2, type I collagen, and CaSR decreased to control levels by NPS2143. There was no significant difference in the expression levels of these markers throughout the study period. Furthermore, no significant difference was observed between treatment with the NPS2143 alone and the control levels.

Effect of a CaSR antagonist on osteoblastic protein level of a CaSR in the presence of MTA

MC3T3-E1 cells were cultured with MTA for 1, 2, and 3 days in α-MEM containing 1.0 μM NPS2143, a CaSR antagonist (Fig. 7). The MTA-induced protein level of CaSR decreased to control levels by NPS2143. There was no significant difference in the expression level of CaSR throughout the study period. Furthermore, no significant difference was observed between treatment with the NPS2143 alone and the control levels.

Discussion

Several studies have reported that elevated extracellular calcium ion levels induce osteoblast differentiation, chemotaxis, proliferation, and mineralization (18,19). Comparable effects have been observed in bone marrow-derived progenitor cells (21), preadipocytes (22), and periodontal ligament cells (17). These findings suggest the presence of a mechanism involving CaSR expression on the cell membrane responding to local changes in calcium ion concentrations.

MTA is a bioactive dental material and a calcium-releasing cement (10-12). Although the release of calcium ions from MTA may cause an interaction between MTA and the surrounding tissues, the pathway by which CaSR contributes directly to the regulation of osteoblast differentiation via calcium ion released from MTA remains unclear. In this study, it was investigated whether MTA could enhance osteogenic gene expression and protein production via CaSR during the early stage of osteoblast differentiation.

MC3T3-E1 cells were used in this study. This cell line is widely used as a model system for osteoblasts because it exhibits properties of osteoprogenitor cells and preosteoblasts (23). Also, many *in vitro* studies using MC3T3-E1 cells have been reported on the characteristics of CaSR in bone physiology (18,19).

Initially, the effect of MTA on cell proliferation and ALP gene expression was examined to assess the biocompatibility of MTA with MC3T3-E1 cells. Cell numbers in the MTA and control groups increased in a time-dependent manner. Although the effect of MTA on MC3T3-E1 cells was minimal, a significant difference between the groups was found on day 3. With similarity to cell numbers, the level of ALP mRNA expression was not inhibited by the presence of MTA, and the significant higher expression was observed in MTA group on day 3, compared to that of control. Those results were consistent with previous reports that MTA enables proliferation (4-8) and ALP expression (24-26) of various cell types, and the excellent biocompatibility of MTA was confirmed in this culture system.

When MC3T3-E1 cells were cultured in the presence of MTA, the gene expression levels of Runx2 and type I collagen increased significantly on days 2 and 3 compared with the control group. Correspondingly, the protein levels were also enhanced in the presence of MTA on day 3. It has been reported that MTA enhances levels of several osteogenic markers in various cell types (2), and present results were largely consistent with these findings. Osteoblast differentiation is controlled by transcription factors including Runx2. Runx2-knockout mice showed defective bone formation and a lack of mature osteoblasts, suggesting that Runx2 is necessary for osteoblast differentiation (27,28). Additionally, a Runx regulatory element is found in the promoter of the type I collagen gene, indicating that the expression of type I collagen is regulated directly by Runx2 (29). Thus, expression levels of Runx2 and type I collagen are related and are considered representative and suitable markers for the assessment of osteoblast differentiation.

In this study, the effect of MTA in early stage of osteoblast differentiation was examined. Runx2 enhances osteoblast differentiation and is also involved in the production of bone matrix proteins at an early stage (23). Additionally, type I collagen mRNA expression and collagen biosynthesis parallel DNA synthesis in immature osteoblasts are reported (30). The present results of a significant increase in both the number of cells and the osteogenic differentiation in day 3, when treated with MTA, may be supported by these reports (23,30).

Interestingly, the gene expression level of CaSR also significantly increased on day 1 of culture in the presence of MTA. The protein levels of CaSR significantly increased on days 2 and 3. These suggest that MTA induces the gene expressions and protein productions of not only osteogenic markers but also CaSR, a sensor of extracellular calcium ions levels.

The calcium ion concentration in the culture medium continuously increased in a time-dependent manner. The α-MEM used for the culture medium already contains 1.8 mM calcium ion, almost the same as that in control group. The increase in the concentration of calcium ions released from MTA over 3 days was ~0.7 mM and the average of increment of each day during experimental periods was ~0.3 mM in this culture system. Recently, osteoblast proliferation through CaSR and activation of phospholipase C in the condition of 2 mM extracellular calcium ions has been shown in primary rat calvarial cells (31). Therefore, it was supposed that the calcium ions released from MTA might affect gene expressions of Runx2, type I collagen via CaSR.

To confirm the specific effect of calcium ions from MTA on the levels of Runx2 and type I collagen, EGTA (0.3 mM), a calcium chelator, was added to the medium in the presence of MTA. The calcium ion concentrations with EGTA alone were significantly lower than those of control during experimental periods. This may be caused by the chelate effect of EGTA. On the other hand, the calcium concentrations with MTA and EGTA were lower than control in day 1, and no significant difference was found in days 2 and 3. These may be attributed to the continuous calcium ion release from MTA. Levels of Runx2 and type I collagen gene expressions in the presence of MTA and EGTA decreased to control levels, with no significant difference between groups. These findings suggest that MTA-induced expression of these genes analyzed in this study may be caused by the continuous release of calcium ions from MTA.

MC3T3-E1 cells were cultured in α-MEM containing 1.0 μM NPS2143, a CaSR antagonist, in the presence of MTA to confirm the involvement of CaSR in MTA-induced gene expression. NPS2143 is an allosteric antagonist that antagonizes the stimulatory effects of calcium ions on CaSR (32). As expected, the calcium ion concentrations with NPS2143 were not affected in the presence or absence of MTA and NPS2143 blocked the stimulatory effect of MTA on levels of Runx2 and type I collagen. This indicated that their levels may be associated with CaSR expression on MC3T3-E1 cell surface.

The gene expression level of CaSR in the presence of MTA decreased to control levels in medium containing EGTA or NPS2143. Also, NPS2143 decreased the protein level of CaSR in the presence of MTA. It has been demonstrated previously that exogenous calcium stimuli increased the expression of CaSR in mammary gland epithelial cells (33) and periodontal ligament cells (17). These findings were consistent with present results. Thus, present data suggest that calcium ions released from MTA affect the gene expression and protein production of CaSR in MC3T3-E1 cells and that the increase of CaSR may be important for the enhancement of MTA-induced osteogenic markers.

In the present study, a cell culture insert system was used to prevent direct contact between MTA and the cells to mimic the clinical condition after filling with MTA. Basically, a medium change was required for cellular growth after a few days. Thus, it is not desirable to use the system in this study for long-term culture of cells, because calcium ions were released continuously from the MTA and accumulated in the medium. However, the present culture system may be suitable for the assessment of MTA-induced osteoblast differentiation during the early stages.

Conclusions

The release of calcium ions from MTA directly promoted the expression of osteogenic genes via CaSR. Additionally, the expression of CaSR was increased by calcium ions released from MTA. The present results help clarify one mechanism of MTA bioactivity and also provide information relevant to the development of endodontic cements.

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Table and Figures

Table 1 Real-time PCR primers used in the experiments

Target	Forward primer	Reverse primer	GenBank Acc.
ALP	5’-GCAGTATGAATTGAATCGGAACAAC-3’	5’-ATGGCCTGGTCCATCTCCAC-3’	NM_007431.32
Runx2	5’-CACTCTGGCTTTGGGAAGAG-3’	5’-GCAGTTCCCAAGCATTTCAT-3’	NM_001145920.1
type I collagen	5’-TGGGCGCGGCTGGTATGAGTTC-3’	5’-ACCCTGCTACGACAACGTGCC-3’	NM_007743.2
CaSR	5’-ATGCTATGGCCCACAGGAACTC-3’	5’-CAGGGCCTGGTGTCTGTTCA-3’	NM_013803.2
GAPDH	5’-AAATGGTGAAGGTCGGTGTG-3’	5’-TGAAGGGGTCGTTGATGG-3’	NM_008084.2

ALP, alkaline phosphatase; Runx2, runt-related transcription factor2; CaSR, calcium-sensing receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

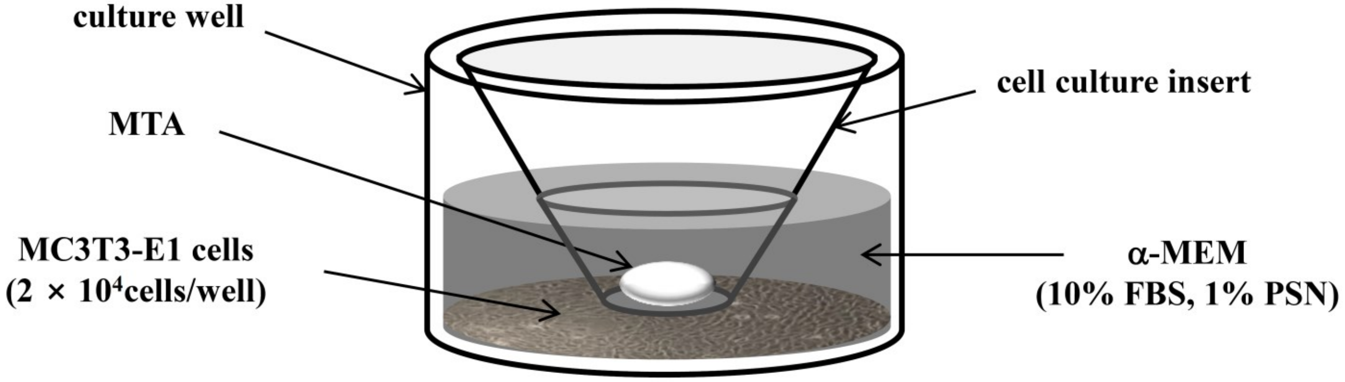

Fig. 1 Culture well with cell culture insert used in this study.
The cell culture insert was placed over the MC3T3-E1 cells, and MTA was placed on the bottom of the cell culture insert.

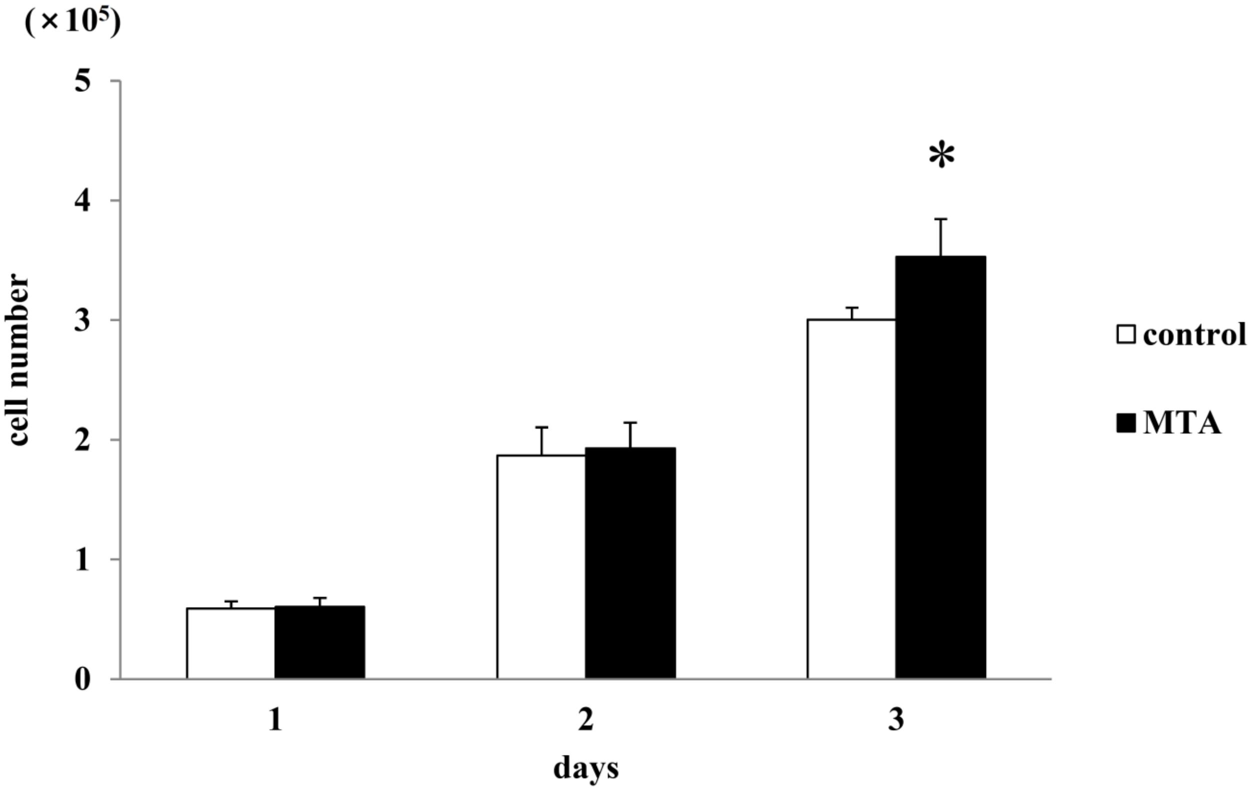

Fig. 2 Effect of MTA on the number of cultured cells.
MC3T3-E1 cells were cultured in 6-well plates up to 3 days with or without (control) MTA. The number of cells was determined by using a Cell Counting Kit 8 on days 1, 2, and 3 of the culture period. Data are mean ± SD for three separate experiments. *$P < 0.05$, MTA treatment versus control.

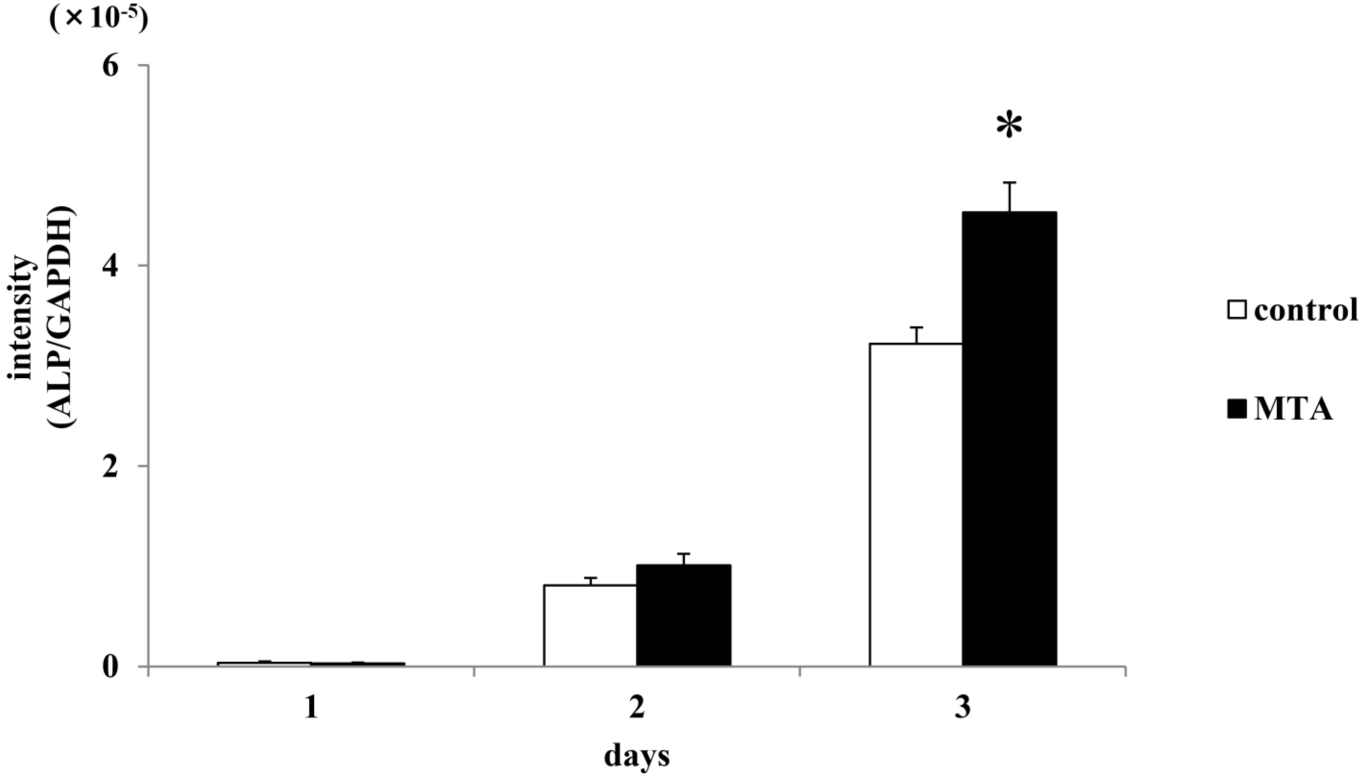

Fig. 3 Effect of MTA on alkaline phosphatase gene expression.

MC3T3-E1 cells were cultured as described in Figure 1. The mRNA levels for ALP were determined by real-time PCR on days 1, 2, and 3 of the culture period. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, MTA treatment versus control.

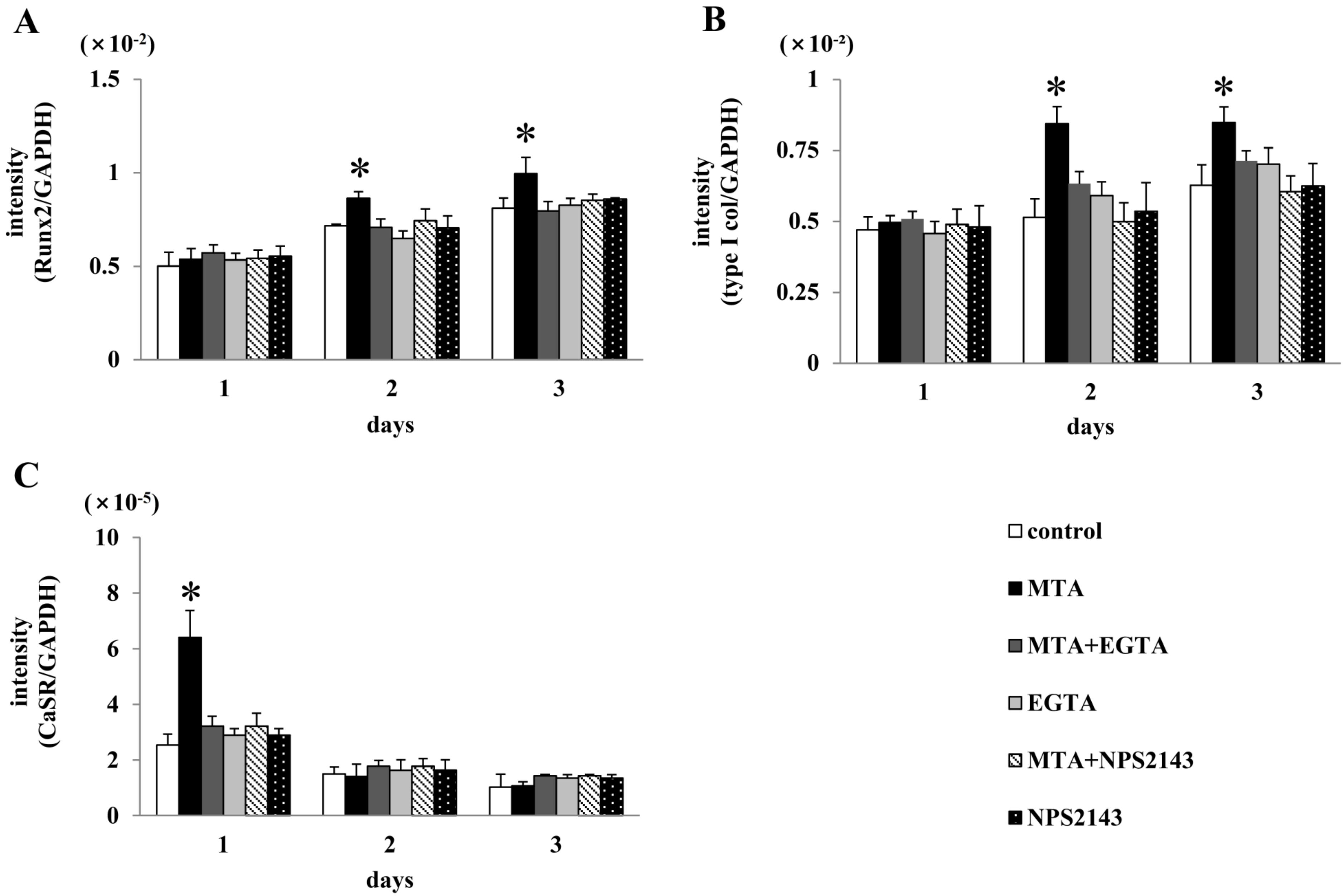

Fig. 4 Effect of MTA on (A) Runx2, (B) type I collagen, and (C) CaSR in the presence or absence of EGTA or NPS2143

MC3T3-E1 cells were cultured as described in Figure 1. The mRNA levels were determined by real-time PCR on days 1, 2, and 3 of the culture period. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, MTA, MTA+EGTA, EGTA, MTA+NPS2143, or NPS2143 treatments versus control.

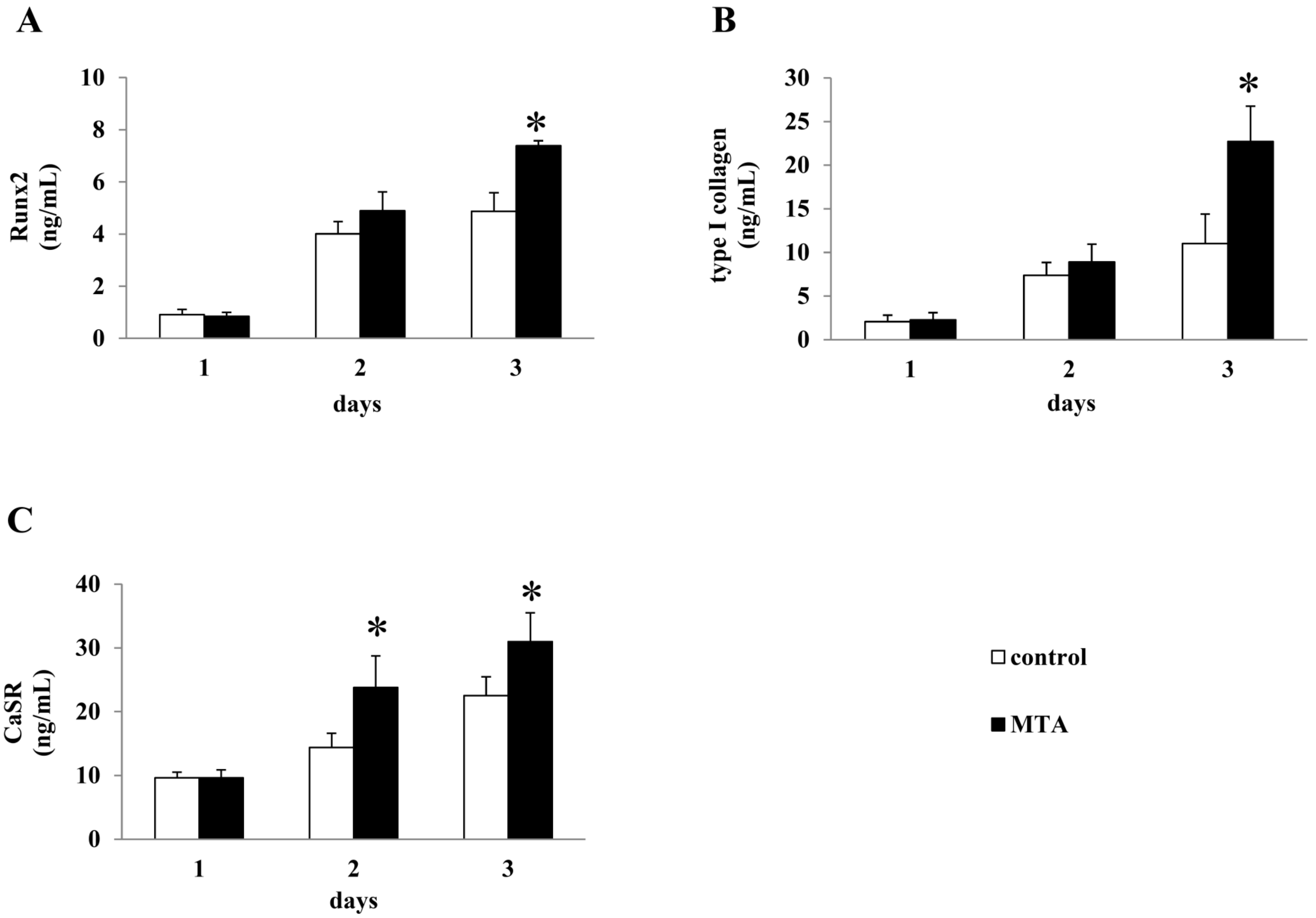

Fig. 5 Effect of MTA on the protein levels of (A) Runx2, (B) type I collagen, and (C) CaSR.
MC3T3-E1 cells were cultured as described in Figure 1. The protein levels were determined by ELISA on days 1, 2, and 3 of the culture period. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, MTA treatment versus control.

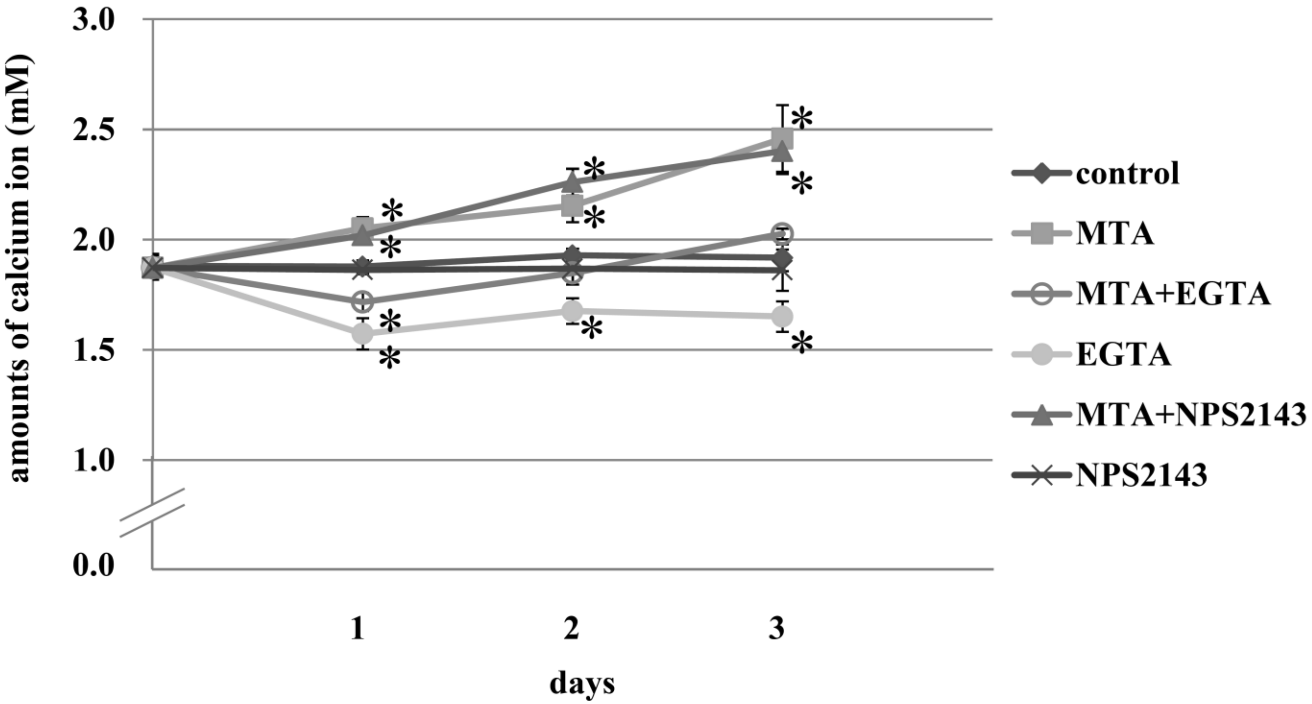

Fig. 6 Calcium ions released from MTA into the culture medium.
Data are mean ± SD for three separate experiments. *$P < 0.05$, MTA, MTA+EGTA, EGTA, MTA+NPS2143, or NPS2143 treatments versus control.

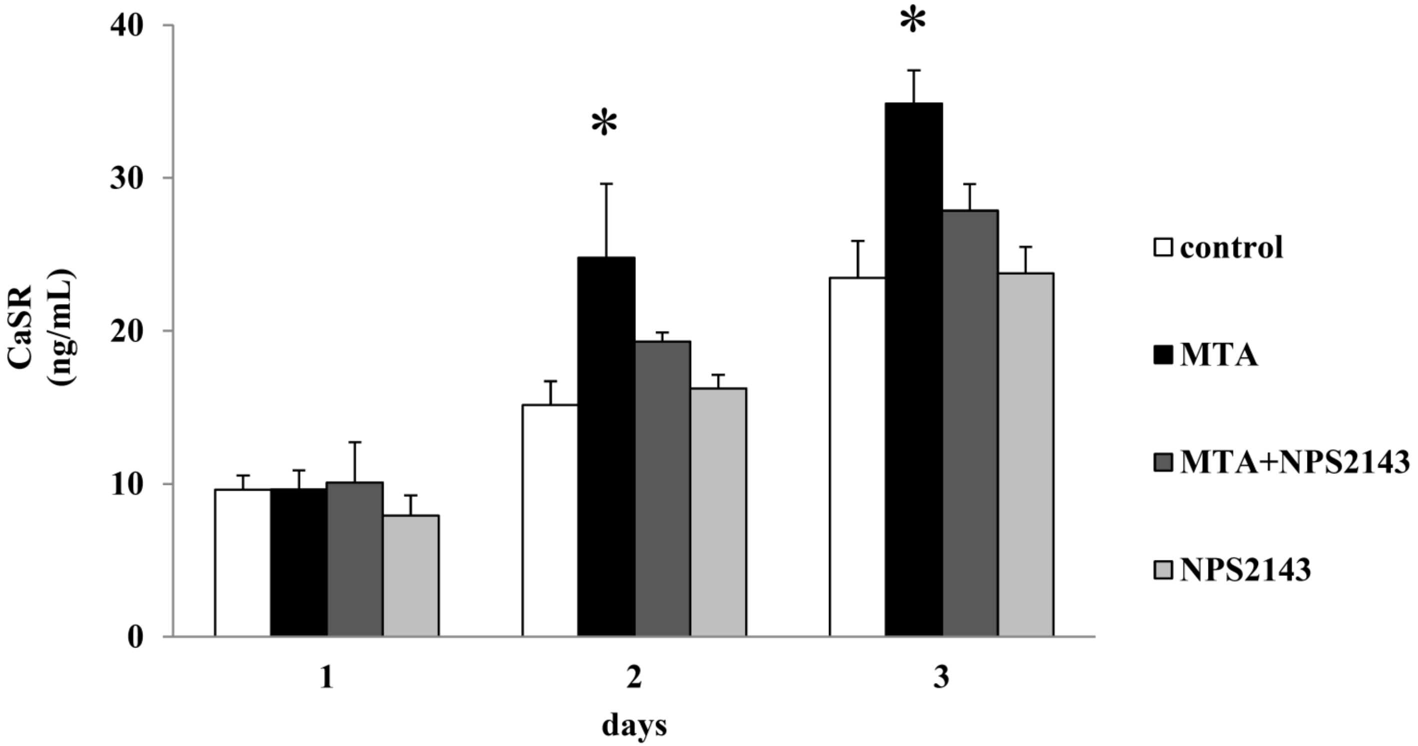

Fig. 7 Effect of NPS2143 and/or MTA on the protein level of CaSR.
MC3T3-E1 cells were cultured as described in Figure 1. The protein levels were determined by ELISA on days 1, 2, and 3 of the culture period. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, MTA, MTA+NPS2143, or NPS2143 treatments versus control.