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ORIGINAL ARTICLE

Camphoric acid stimulates osteoblast differentiation and induces glutamate receptor expression

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Abstract We found that camphoric acid significantly stimulated the differentiation of mouse osteoblastic MC3T3-E1 subclone 4 cells, as indicated by the induction of markers of osteoblastic differentiation. To elucidate the mechanism of action of camphoric acid in osteoblast differentiation, we evaluated the induction of transient receptor potential (TRP) cation channel family members and glutamate signaling molecules. TRPM7 and TRPV1 were highly expressed, but their expression was unaffected by camphoric acid. Camphoric acid is structurally similar to glutamate receptor ligands and significantly induced the expression of NMDAR1, GluR3/4, and mGluR8. However, camphoric acid exhibited weak regulatory activity toward glutamate receptors in a radioligand binding assay. Camphoric acid also significantly induced the activation of NF-κB and AP-1. Together, these data suggest that the stimulatory effect of camphoric acid on osteoblast differentiation was the result of its ability to induce mRNA expression of glutamate signaling molecules and to activate transcription factors.

Keywords Camphoric acid · Osteoblastic cells · Differentiation · Glutamate receptors · Transcription factors

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Introduction

Bone tissue is continuously remodeled through a cycle of destruction and rebuilding (Harada and Rodan 2003). The process by which old bone is removed by multinucleated osteoclasts and new bone is added via mineralization by osteoblasts is referred to as bone remodeling. Usually, a balance between osteoclastic bone resorption and osteoblastic bone formation maintains the bone mass at a homeostatic steady state; however, an imbalance in this process favoring bone resorption over bone formation results in a variety of adult skeletal diseases, including osteoporosis. The development and progression of osteoporosis increases the risk for fractures (particularly in the hip) and their many adverse consequences, including substantial skeletal deformity, pain, and functional limitations (van der Klift et al. 2005).

Until now, most therapies for osteoporosis have focused on limiting resorption, with varying success (Rodan and Martin 2000). Thus, therapies that work by increasing bone mass, improving defects in bone microarchitecture, and accelerating fracture healing are urgently needed.

Natural compounds and their derivatives have been an invaluable source for therapeutic agents, and recent research efforts to develop therapeutic agents that affect bone remodeling have focused on natural substances. A wide variety of natural compounds can influence bone

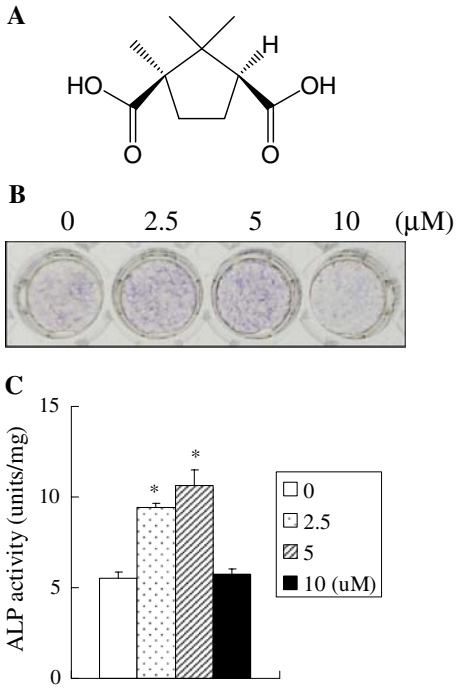


Fig. 1 Chemical structure of camphoric acid (**a**) and its effect on alkaline phosphatase (ALP) expression and activity on differentiation day 9. Differentiation was induced in the absence or presence of camphoric acid. On day 9, ALP protein expression was evaluated by ALP staining (**b**) and by measuring ALP activity (units/mg). The assays were performed in triplicate as described in "Materials and methods" (**c**). The data are expressed as the mean ± standard deviation. $*P < 0.001$ as compared with vehicle control

remodeling, particularly by inhibiting bone resorption, thereby having beneficial effects on the skeleton (Putnam et al. 2007). However, the ability of natural compounds to stimulate osteoblast differentiation has been reported in several recent studies (Lee et al. 2007; Jeong et al. 2008). Previously, we showed that (1R,3S)-(+)-camphoric acid (Fig. 1a) stimulated calcium formation in mouse osteoblastic MC3T3-E1 subclone 4 cells. Camphoric acid is obtained from the oxidation of camphor and is commonly used for its topical antipruritic, analgesic, and counterirritant properties (Burkhart and Burkhart 2003). Camphoric acid acts specifically upon the skin and mucous membranes to control excessive secretion. It is particularly effective for treating night sweating, e.g., due to tuberculosis, but is also valuable for controlling the profuse sweating that occurs during recovery from prostrating fevers. The biological effect of camphoric acid on bone metabolism has not been studied. Based on our preliminary experiments showing the stimulating effect of camphoric acid on bone formation, we evaluated the effect of camphoric acid on osteoblast differentiation and investigated the mechanism of its action.

Materials and methods

Cell culture

All materials for cell culture were purchased from HyClone (Logan, UT, USA). Mouse osteoblastic MC3T3-E1 subclone 4 cells (hereafter referred to as MC3T3-E1 cells) with high differentiation potential (Wang et al. 1999) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 mg/ml of streptomycin [growth medium (GM)] in humidified atmosphere of 5% CO_2 at 37°C. The culture medium was changed every 3 days.

Osteoblast differentiation

Cells suspended in GM were plated in 24-well plates. After reaching confluence, cells were cultured in differentiation medium [DM: GM with 50 μg/ml of ascorbic acid (Fluka, Sigma–Aldrich Laborchemikalien GmbH, Seelze, Germany) and 10 mM of β-glycerophosphate (Sigma, St Louis, MO, USA)]. The medium was changed every 3 days.

Alkaline phosphatase staining and activity assay

Cells were cultured in DM in the presence or absence of camphoric acid (purity > 99%, Sigma); the medium was changed every 3 days. On differentiation day 9, cells were washed twice with phosphate buffered saline (PBS, HyClone), fixed with 10% formalin, rinsed with deionized water, and stained under protection from direct light using Alkaline Phosphatase kit (Sigma). To measure alkaline phosphatase (ALP) activity, cells were washed twice with PBS and sonicated in lysis buffer (10 mM Tris–HCl, pH 7.5, 0.5 mM $MgCl_2$ and 0.1% Triton X-100). After the centrifugation at 10,000×g for 20 min at 4°C, the supernatant was transferred and the ALP activity was measured by using LabAssay ALP kit (Wako Pure Chemicals Industries, Osaka, Japan). The protein concentration of each sample was measured using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Activity assays were performed in triplicate and the significance of differences was determined by Student's t test. Differences were considered significant when $P < 0.001$.

Alizarin red S and von Kossa staining

Cells were washed twice with PBS, stained with 40 mM of Alizarin red S solution (pH 4.2) for 10 min at room temperature, and washed twice with deionized water. For

von Kossa staining, cells were washed with PBS and fixed with 2.5% glutaraldehyde in PBS for 30 min. After washing with deionized water three times, cells were incubated with 5% silver nitrate at room temperature under UV light until the calcium turned black. After washing with deionized water three times, the images of stained cells were captured under a microscope fitted with a DP70 digital camera.

Measuring calcium content

To measure calcium content, cells were washed twice with PBS, fixed with 3.7% formaldehyde in PBS for 15 min, and decalcified with 300 µl of 1 N HCl for 24 h. The amount of calcium was then measured using the Calcium C kit (Wako Pure Chemicals Industries) according to the manufacturer's protocol.

Computational molecular modeling

Computational molecular modeling studies were performed in order to identify common structural features between the camphoric acid and glutamate receptor antagonists and agonists. The structures for camphoric acid and the compounds extracted from the known glutamate receptors retrieved from the protein data bank were submitted to energy minimization and conformational analysis using the ConFirm program implemented into Catalyst (http://www.accelrys.com/products/catalyst/). Using the HipHop algorithm of Catalyst, pharmacorphore models for camphoric acid were built. The extracted compounds from protein data bank were mapped against the pharmacorphore model for camphoric acid and the fit values for each compound were obtained. We also calculated two-dimensional-similarities between camphoric acid and the extracted compounds from protein data bank using the Tanimoto constant. As shown in Table 4, we chose five kinds of compounds based on the glutamate receptor structures, mGluR1 [1ISS.pdb; (*S*)-alpha-methyl-4-carboxyphenylglycine (MCPG)], GluR2 [1FTK.pdb; 3-(carboxymethyl)-4-isopropentylproline (KAI)], GluR6 [1S9T.pdb; (*S*)-2-amino-3-(3,5-dioxo-[1,2,4]oxadiazolidin-2-yl)-propionic acid (QUS)], GluR5 [2F34.pdb; (*S*)-1-(2-amino-2-carboxyethyl)-3(2-carboxythiophene-3-yl-methyl)-5-methylpyridine-2,4-dione (UBA)], and NMDAR1 [1Y1 M.pdb; 1-aminocyclopehntanecarboxylic acid (AC5)], and then carried out docking studies using FlexX method implemented by Sybyl7.3 (Hindle et al. 2002).

Radioligand binding assays

Radioligand binding assays were performed by MDS Pharma Services (Taipei, Taiwan) to determine the receptor selectivity of camphoric acid at 10 µM for eight glutamate receptors.

Evaluation of mRNA expression

Primers were designed using an on-line primer design program (Rozen and Skaletsky 2000). The sequences of the primers used in this study are shown in Table 1. Total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to manufacturer's protocol. The concentration and purity of total RNA were determined by measuring absorbance at 260 and 280 nm. First-strand cDNA was synthesized using 1 µg of total RNA, 1 µM of oligo-dT_{15} primer, and Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). SYBR green-based quantitative PCR was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) with first-strand cDNA diluted 1:50 and 20 pmole of primers, according to the manufacturer's protocols. The PCR consisted of initial denaturation at 94°C for 3 min, three-step cycling (40 cycles) at 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All product signals were normalized to the GAPDH signal, which served as an internal control. The significance of differences was determined by Student's *t* test. Differences were considered significant when $P < 0.05$.

Transient transfection and dual-luciferase reporter assay

Cells were plated in 96-well plates at 5×10^3 cells/well. Once cells reached 90–95% confluency, they were transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Each transfection contained 200 ng/well each of NF-κB and AP-1-firefly luciferase reporter plasmid (Clontech, Mountain View, CA, USA) plus 20 ng/well of pGL4.73-*Renilla* luciferase (Promega, Madison, WI, USA). Five hours later, cells were treated with camphoric acid in DM. After 24 h, the transfected cells were lysed and the firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase Reporter Assay kit (Promega). Firefly luciferase activity was normalized to the *Renilla* luciferase activity. Data are presented as the relative ratios of test: control normalized luciferase activities. DMSO was used as a vehicle control. The significance of differences was determined by Student's *t* test; differences were considered significant when $P < 0.05$.

Table 1 Primer sequences

Target gene	Forward (5′–3′)	Reverse (5′–3′)
Osteocalcin	AAGCAGGAGGGCAATAAGGT	TTTGTAGGCGGTCTTCAAGC
Osteopontin	CGATGATGATGACGATGGAG	TGGCATCAGGATACTGTTCATC
Type I collagen	ACGTCCTGGTGAAGTTGGTC	CAGGGAAGCCTCTTTCTCCT
NMDAR1	ACTCCCAACGACCACTTCAC	GTAGACGCGCATCATCTCAA
NMDAR2A	AGACCTTAGCAGGCCCTCTC	CTCTTGCTGTCCTCCAGACC
NMDAR2B	CCGCAGCACTATTGAGAACA	ATCCATGTGTAGCCGTAGCC
NMDAR2C	GCAGAACTTCCTGGACTTGC	CACAGCAGAACCTCCACTGA
NMDAR2D	CCATCGAGCCTTCTTGTCAT	ACCATGAACCAGACGTAGCC
NMDAR3B	CTACATCAAGGCGAGCTTCC	AGCTTGCAGTCCGCATCTAT
GluR1	CTGTGAATCAGAACGCCTCA	AGCGTCACTTGTCCTCCACT
GluR2	GGAGGTGTGTGGTGGTTCTT	AGGGCTCTGCACTCCTCATA
GluR3	GACCCTGGACTCTGGTTCAA	ATCACACGGCTTTCTCTGCT
GluR4	CCGAAACACAGACCAGGAAT	GAGCACTGCAGAAGGAGGTC
KA1	TAATGCTGAAGGGGAACCAC	ACTTTCTCACGTTCCGCTGT
mGluR1	CCCTTTACAACGTGGAGGAA	GAACAAGGGCGTCTCTTCTG
mGluR4	CCTCAGAATAGAGCGGATGC	CATGTCATAGGGGCAGGTCT
mGluR8	GCCACTGGACCAATCAACTT	GGGCAGAGTTCACAGGAGAG
GLAST	CCAAAAGCAACGGAGAAGAG	ACCTCCCGGTAGCTCATTTT
PSD-95	CTATGAGACGGTGACGCAGA	CGGGAGGAGACAAAGTGGTA
TRPV3	CGAGCTGTGCAAAGTAGCAG	CAAACGCATAGAGGGTGGTT
TRPC1	ACCTTCCACTCGTTCATTGG	GCTCGAGCAAACTTCCATTC
TRPC3	GTAGTGGGTGTGCTGGACCT	AGCCTGCTACAAGGTGCAAT
TRPC4	GACACGGAGTTCCAGAGAGC	GACCTGTCGATGTGCTGAGA
TRPC6	GGTGCGGAAGATGCTAGAAG	GCTTGTCGCTAACCTTTTGC
TRPV1	GTGACCCTCTTGGTGGAGAA	CTTCAGTGTGGGGTGGAGTT
TRPV3	CCCCATCCTCTTTCTCTTCC	ACGCATAGAGGGTGGTTTTG
TRPV5	TTGGTGCCTCTCGCTACTTT	AGCGCAGTAGGTCTCCAAAA
TRPV6	GCACTGTTCAGCACCTTTGA	GACCATACTCTCGCCCACAT
TRPM6	CAGTGGTCTGTGGAGAAGCA	CCTGGCTGAAGGTCTCTTTG
TRPM7	GCTCCATGGGGAGTGATAGA	ATCAAAGCCACCACAGGAAC
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Results

We evaluated the effect of camphoric acid on the expression and activity of ALP, an early marker of osteoblast differentiation. Camphoric acid (up to 5 μM) significantly stimulated ALP expression and activity on differentiation day 9 as compared to control (Fig. 1b, c). At 10 μM, we could discern no effect on ALP activity relative to the control.

On differentiation day 15, we evaluated the effect of camphoric acid on mineral deposition by staining with Alizarin red S (Fig. 2a, upper image) and measuring the amount of deposited calcium (Fig. 2b). Continuous treatment with camphoric acid (up to 5 μM) strongly accelerated calcium formation. On differentiation day 18, the effect of camphoric acid on mineralization was evaluated by von Kossa staining (Fig. 2a, lower image), and we found that mineralization was induced by low concentrations of camphoric acid; the staining pattern was consistent with that of Alizarin red S on day 15.

In order to determine whether camphoric acid had an anabolic affect on osteoblasts, we evaluated the expression of osteoblast mineralization-related genes, such as osteocalcin, osteopontin, and type I collagen, on differentiation day 9. Quantitative real-time PCR showed that camphoric acid significantly increased the expression of osteocalcin, osteopontin, and type I collagen mRNA in a dose-dependent manner (Table 2).

In order to determine the mechanism of camphoric acid's anabolic effects, we evaluated the effect of camphoric acid on the expression levels of ten transient receptor potential (TRP) cation channel family members (TRPC1/3/4/6, TRPM6/7, and TRPV1/3/5/6) in MC3T3-E1 cells on differentiation day 9. Camphor has been

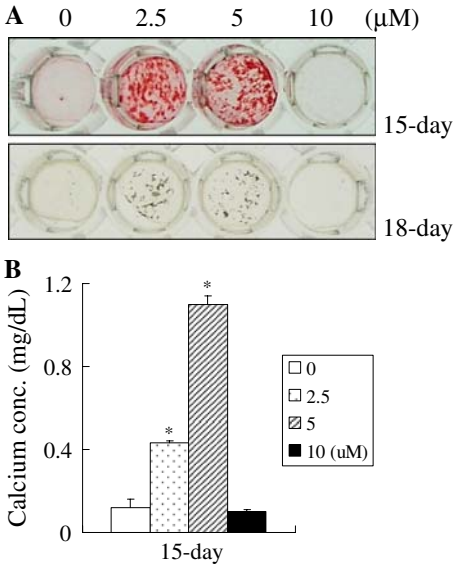


Fig. 2 Effect of camphoric acid on calcium formation. On differentiation day 15, mineralized nodule formation was evaluated by Alizarin red S staining (**a** *upper image*) and the amount of newly synthesized calcium was measured in triplicate as described in "Materials and methods" (**b**). The data are expressed as the mean ± standard deviation. * $P < 0.001$ as compared to vehicle control. On day 18, camphoric acid-induced mineralization was visualized by von Kossa staining (**a** *lower image*)

reported to activate or inhibit several TRP channel subfamily members (Moqrich et al. 2005; Xu et al. 2005). In this study, we found that TRPM7 and TRPV1 were highly expressed in MC3T3-E1 cells, but osteoblast differentiation and the addition of camphoric acid had no effect on the expression levels of either protein (Table 3). Amplification of TRPC3 and TRPV6 revealed a shifted band and three major bands that could correspond to different splicing variants in osteoblasts; however, the expression of TRPC1/4/6 and TRPV3/5 was not detected by RT-PCR (data not shown).

Camphoric acid and ligands of glutamate receptors exhibit structural similarities; therefore, we used computational molecular modeling to evaluate whether camphoric acid could activate glutamate receptors. As shown in Table 4, although binding scores for GluR6 and NMDAR1 were somewhat low, camphoric acid was able to dock in the active site of various glutamate receptors, regardless of the isozyme type. Therefore, we evaluated the effect of camphoric acid on the regulation of glutamate receptors by performing a radioligand binding assay and found that camphoric acid exhibited very weak activity in all cases (less than 20% at 10 μM; Fig. 3a). We further evaluated the effect of camphoric acid on the expression of glutamate signaling molecules (NMDAR1/2A/2B/2C/2D/3B, GluR1/2/3/4, KA1, mGluR1/4/8, GLAST, and PSD-95) reported to be involved in osteoblast function by quantitative real-time PCR (Fig. 3b). In MC3T3-E1 cells, NMDAR1/2C, GluR3/4, mGluR8, GLAST, and PSD-95 were expressed as in the mouse brain and, interestingly, camphoric acid significantly induced the expression of NMDAR1, GluR3/4, and mGluR8.

The effect of camphoric acid on the activation of transcription factors NF-κB and AP-1 was evaluated by luciferase reporter assay (Fig. 4). As compared to the control, the expression of NF-κB and AP-1 was elevated significantly by the same concentration of camphoric acid that stimulated osteoblast differentiation.

Discussion

We demonstrated that camphoric acid stimulates osteoblast differentiation. In order to elucidate the mechanism by which camphoric acid induces differentiation, we evaluated its effect on the expression of TRP cation channel family members, because camphor has been reported to activate or inhibit several members of the TRP cation channel subfamily (Moqrich et al. 2005; Xu et al. 2005).

Based on their amino acid sequence similarities, the TRP-related proteins fall into seven subfamilies (Flockerzi 2007): the classical TRPs (TRPCs), which display greatest similarity to Drosophila TRP; the vanilloid receptor TRPs

Table 2 Effect of camphoric acid on the expression of osteoblast differentiation-related genes on differentiation day 15

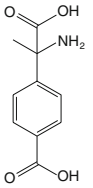
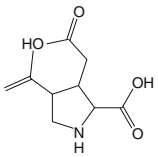
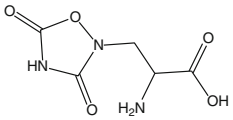
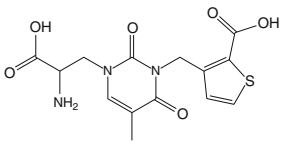
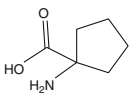
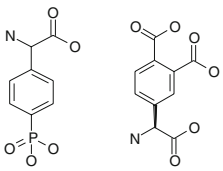
** $P < 0.01$; *** $P < 0.001$

Camphoric acid (μM)	Osteocalcin	Osteopontin	Type I collagen
0	1.19 ± 0.82	1.00 ± 0.21	1.00 ± 0.08
2.5	10.74 ± 1.23**	13.06 ± 0.41**	21.39 ± 5.03***
5	30.88 ± 3.21**	15.12 ± 2.93**	76.83 ± 1.60***

Table 3 Effect of camphoric acid on the expression of TRP cation channel family members on differentiation day 9

	Growth medium	Differentiation medium + camphoric acid (μM)	
		0	5
TRPM7	1.00 ± 0.10	0.92 ± 0.06	1.23 ± 0.13
TRPV1	1.11 ± 0.62	1.24 ± 0.20	1.32 ± 0.25

Table 4 Computational molecular modeling for camphoric acid and other compounds related to glutamate receptor isozymes

PDB ID[a]	Type	Ligand structure	Ligand name	Binding score for ligand	Binding score for camphoric acid	2D-similarity[b]
1ISS	mGluR1; antagonist		(*S*)-alpha-methyl-4-carboxyphenylglycine (MCPG)	−24.6	−17.37	0.65
1FTK	GluR2		3-(carboxymethyl)-4-isopropentylproline (KAI)	−28.86	−14.34	0.76
1S9T	GluR6		(*S*)-2-amino-3-(3,5-dioxo-[1,2,4]oxadiazolidin-2-yl)-propionic acid (QUS)	−35.98	−6.07	0.68
2F34	GluR5; antagonist		(*S*)-1-(2-amino-2-carboxyethyl)-3(2-carboxythiophene-3-yl-methyl)-5-methylpyridine-2,4-dione (UBA)	−29.5	−18.16	
1Y1M	NMDAR1		1-aminocyclopehntanecarboxylic acid (AC5)	−30.14	−8.89	0.05
	mGluR8[c]		2-amino-2-(4-phosphonophenyl) acetic acid (left)/(*S*)-4-(amino(carboxy)methyl) phthalic acid (right)			2.56/0.16

[a] Protein Data Bank identification number

[b] Tanimoto similarity constant

[c] mGluR8 has no PDB structure

(TRPVs); the melastatin TRPs (TRPMs); the mucolipins (TRPMLs); the polycystins (TRPPs); and ankyrin transmembrane proteins 1 (TRPA1). The seventh subfamily, TRPN, comprises proteins in tunicates, flies, and worms; members of this subfamily have not been identified yet in mammals. The biological roles of TRP channels appear to be equally diverse and range from roles in temperature and pain perception to Ca^{2+} and Mg^{2+} absorption, endothelial permeability, smooth muscle proliferation, and gender-specific behaviors. Interestingly, mice lacking TRPV5, which has been shown to be the gatekeeper in active Ca^{2+} resorption in the kidney, exhibited significant disturbances in bone structure, including reduced trabecular and cortical bone thickness (Hoenderop et al. 2003). Recently, TRPCs and TRPM7 were shown to be expressed in human osteoblast-like MG-63 cells and were suggested to be involved in the proliferation of osteoblasts (Abed and Moreau 2007; Labelle et al. 2007). In ROS 17/2.8 rat osteoblastic-like cells, two fragments were amplified (390 and 201 bp) using primers based on conserved regions within the mammalian TRPC3/6/7 subfamily. These fragments had 100 and 94% sequence identity, respectively, with human TRPC3 (Baldi et al. 2003). In the present study, the expression of ten members of TRP cation channel family was evaluated in mouse osteoblastic MC3T3-E1 subclone 4 cells. TRPM7 and TRPV1 were highly expressed; however, the expression levels of neither protein were affected by osteoblast differentiation or by the addition of camphoric acid. Amplification with primers specific for TRPC3 and TRPV6 showed a shifted band and three major bands that might correspond to different splicing variants in osteoblasts; yet, TRPC1/4/6 and TRPV3/5 expression was not detected by

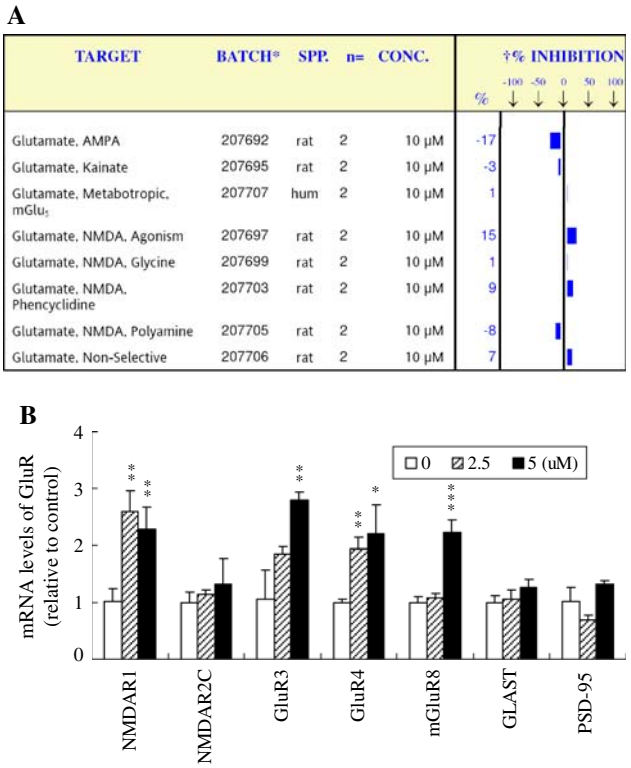


Fig. 3 Effect of camphoric acid on glutamate receptor binding and glutamate receptor expression. **a** In the radioligand binding assay, results are expressed as percentage inhibition with 10 µM of camphoric acid. **b** The effect of camphoric acid on the expression of glutamate signaling molecules was evaluated on differentiation day 9 by quantitative real-time PCR. The GAPDH-normalized fold changes are expressed as the mean ± standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to vehicle control

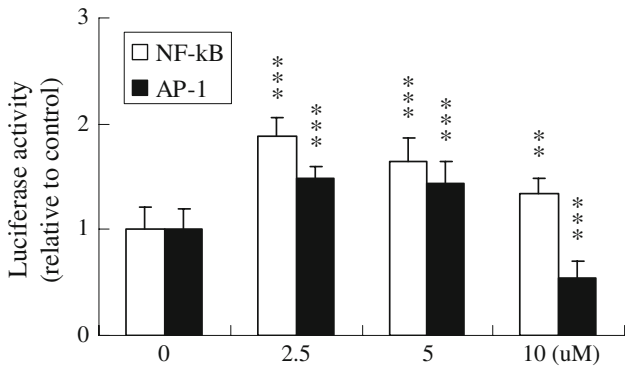


Fig. 4 Effect of camphoric acid on the activation of transcription factors NF-κB and AP-1. The effect of camphoric acid on the activation of NF-κB and AP-1 was evaluated by luciferase reporter assay. Cells were seeded in 96-well plates, transfected with NF-κB and AP-1-firefly luciferase plus pGL4.73-*Renilla* luciferase reporter plasmids. Camphoric acid was added after 5 h. After 24 h, the activities of both types of luciferase were measured. The firefly luciferase activity was normalized to the *Renilla* luciferase activity. Data are presented as the relative ratios of normalized luciferase activity to the control activity. DMSO was used as a vehicle control. ** $P < 0.01$; *** $P < 0.001$ as compared to control

RT-PCR. These data suggest that the induction of TRPs might not be involved in the process of camphoric acid-induced osteoblast differentiation.

The activity of camphoric acid to suppress mucous membrane and sweat gland secretion could also be connected to a blockade of glutamate receptors or glutamate transporters. Mucous membrane secretion could be activated by dicarboxylates including glutamate (Haxhiu et al. 2000). Dicarboxylates are known to inhibit glutamate transporters and this may increase the local availability of glutamate for receptor stimulation (Balcar et al. 1995; Kovacs et al. 1999). Considering the structural similarities between camphoric acid and glutamate signaling receptor ligands, the aforementioned factors made us hypothesize that glutamate signaling receptors might be involved in the effect of camphoric acid on the osteoblast differentiation. Computational molecular modeling indicated that camphoric acid could dock to the active sites of each glutamate receptor, regardless of isozyme type. In cultures of MC3T3-E1 cells grown under osteogenic conditions for up to 6 days, treatment with riluzole, an anticonvulsant that acts by inhibiting glutamate release at central synapses, inhibited ALP activity and glutamate release significantly (Genever and Skerry 2001). These findings support the idea of an intrinsic osteo-glutamatergic signaling mechanism. Additionally, during osteoblastic differentiation of MC3T3-E1 cells over several days in culture, increased levels of glutamate exocytosis, elevated intracellular free glutamate, and increased sensitivity to depolarization-induced inhibition of glutamate release suggested that osteoblasts attain a more active glutamatergic phenotype as they differentiate. This could be explained with the induction of glutamate signaling molecules by osteogenic compounds, such as camphoric acid. However, in this study camphoric acid exhibited very weak regulatory activity toward glutamate receptors. In addition, considering that effective anti-sweating dosages of camphoric acid could be in the range of 10–20 mg/kg that might be above 10 µM in body fluid, camphoric acid might be beneficial to sweating at high concentrations. Since camphoric acid exhibited a peak of stimulation between 2.5 and 5 µM, and a tendency toward an inhibition at 10 µM in this study, low dosages of camphoric acid could be beneficial to osteoblast differentiation.

Several studies have reported the expression of glutamate receptors in osteoblasts; osteoblasts express ionotropic glutamate receptor (iGluR), *N*-methyl-D-aspartate (NMDA), a-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPK), kainate (KA), and metabotropic glutamate receptors (mGluR; Chenu 2002; Mason 2004). Electrophysiological studies showed that NMDARs are functional in osteoblasts (Laketić-Ljubojević et al. 1999; Gu et al. 2002). Signaling proteins that are associated with

the clustering of glutamate receptors (such as PSD-95) and its transporter (such as GLAST) in the central nervous system are also expressed in osteoblasts (Mason et al. 1997; Patton et al. 1998).

In this study, we showed that NMDAR1/2C, GluR3/4, mGluR8, GLAST, and PSD-95 were expressed in MC3T3-E1 subclone 4 cells, as in the mouse brain, and camphoric acid significantly induced the expression of NMDAR1, GluR3/4, and mGluR8. The expression of NMDAR1, GluR3, and mGluR8 in primary cultures of rat calvarial osteoblastic cells and the decreased expression of NMDAR1 in disused bones in a rat model of osteopenia suggest that these molecules contribute to osteogenesis (Hinoi et al. 2001, 2002; Ho et al. 2005). Northern analysis confirmed the expression of GLAST in bone and in situ hybridization localized GLAST mRNA to osteoblasts and osteocytes (Mason et al. 1997). Interestingly, on quiescent periosteal surfaces, GLAST expression was almost absent, while on surfaces where loading had induced cellular proliferation and bone formation GLAST protein expression was elevated, suggesting that the manipulation of bone function by moderators of glutamate action could provide novel treatments for bone diseases such as osteoporosis. Additionally, the NMDAR clustering protein PSD-95 was shown to be expressed in rat bone cells (Patton et al. 1998) and sustained exposure to the NMDA channel antagonist significantly prevented increases in both ALP activity and Ca^{2+} accumulation in a dose-dependent manner in osteoblasts (Hinoi et al. 2003). Together with our data, these findings suggest that the heteromeric NMDA receptor channels are expressed to regulate mechanisms that underlie cellular differentiation rather than proliferation and/or maturation.

The effect of camphoric acid on the induction of glutamate signaling molecules prompted us to evaluate its effect on the activation of transcription factors, such as NF-κB and AP-1, known to be important in the regulation of gene expression during osteoblast differentiation. The induction of AP-1 and coordinated regulation of NF-κB and AP-1 influence the regulation of the osteopontin gene (Kim et al. 2002; Renault et al. 2005). Interestingly, several osteoblast-specific genes could be regulated via AP-1 binding sites in their promoters (Stein et al. 1996; Narayanan et al. 2004; Sakata et al. 2004), and there is accumulating evidence that members of AP-1 complex participate in the regulation of cell proliferation, differentiation, and maturation in bone. Recently, AP-1 was reported to be a potent regulator of NMDAR2B and GLAST at the level of transcription (Qiang and Ticku 2005; Ramírez-Sotelo et al. 2007), suggesting the induction of glutamate signaling molecules by the activation of transcription factors.

In summary, we demonstrated that NMDAR1/2C, GluR3/4, mGluR8, GLAST, and PSD-95 were expressed in mouse osteoblastic MC3T3-E1 subclone 4 cells. Camphoric acid induced the expression of NMDAR1, GluR3/4, and mGluR8, suggesting that the stimulatory effect of camphoric acid on osteoblast differentiation which resulted from its potential to induce the expression of glutamate signaling molecules (especially, NMDAR1, GluR3/4, and mGluR8) in mouse osteoblastic cells. Additionally, although further studies are needed to elucidate the relationship between the activation of transcription factors and the induction of glutamate signaling molecules by camphoric acid, our data suggest that camphoric acid-mediated transcription factor activation might be associated with of the ability of camphoric acid to induce glutamate signaling molecules, leading to osteoblast differentiation.

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