Low-Frequency Pulsed Electromagnetic Fields Promote Osteoblast Differentiation via the cAMP/PKA Signaling Pathway: A Postprint

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Abstract

Objective To investigate whether 50 Hz 0.6 mT low-frequency pulsed electromagnetic fields (PEMFs) promote osteoblast differentiation via the cAMP/PKA signaling pathway.

Methods Rat calvarial osteoblasts (ROBs) were cultured in vitro. After passaging and reaching confluence, cells were exposed to 50 Hz 0.6 mT low-frequency PEMFs for various durations, and intracellular cAMP concentration and PKA phosphorylation levels were measured. 2',3'-Dideoxyadenosine (DDA) was used to inhibit intracellular adenylyl cyclase (AC) activity, and changes in alkaline phosphatase (ALP) activity and osteogenic gene transcription following PEMF stimulation were detected. KT5720 was employed to inhibit PKA phosphorylation, and alterations in osteogenic gene transcription and protein expression after PEMF stimulation were examined.

Results Following 20 min of exposure to 50 Hz 0.6 mT PEMFs, intracellular cAMP concentration in ROBs increased significantly, persisted until 40 min, then declined rapidly, and increased again at 2 h. p-PKA exhibited a similar pattern, indicating that PEMFs activated the cAMP/PKA signaling pathway. Inhibition of adenylyl cyclase activity with DDA markedly attenuated the PEMF-induced elevation of ALP activity and osteogenic gene transcription. Similarly, inhibition of PKA phosphorylation with KT5720 reduced PEMF-induced osteogenic gene transcription and protein expression, demonstrating that the cAMP/PKA signaling pathway participates in PEMF-promoted ROB differentiation.

Conclusion 50 Hz 0.6 mT low-frequency PEMFs promote ROB differentiation through the cAMP/PKA signaling pathway.

Full Text

Abstract

Objective: To investigate whether 50 Hz 0.6 mT low-frequency pulsed electromagnetic fields (PEMFs) promote osteoblast differentiation through the cAMP/PKA signaling pathway. **Methods:** Rat calvarial osteoblasts (ROBs) were cultured in vitro and exposed to 50 Hz 0.6 mT PEMFs for varying durations after reaching confluence. Intracellular cAMP concentration and PKA phosphorylation levels were measured. The adenylate cyclase (AC) inhibitor 2' ,3' -dideoxyadenosine (DDA) was used to suppress AC activity, and changes in alkaline phosphatase (ALP) activity and osteogenic gene transcription were assessed following PEMF stimulation. The PKA inhibitor KT5720 was employed to block PKA phosphorylation, and its effects on PEMF-induced osteogenic gene transcription and protein expression were examined. **Results:** After 20 min of PEMF exposure, intracellular cAMP concentration increased significantly, remained elevated until 40 min, then declined rapidly before rising again at 2 h. Phosphorylated PKA (p-PKA) exhibited a similar pattern, demonstrating that PEMFs activate the cAMP/PKA signaling pathway. Inhibition of AC activity with DDA significantly attenuated the PEMF-induced increases in ALP activity and osteogenic gene transcription. Similarly, suppression of PKA phosphorylation with KT5720 reduced PEMF-induced osteogenic gene transcription and protein expression, confirming that the cAMP/PKA pathway mediates PEMF-promoted ROB differentiation. **Conclusion:** 50 Hz 0.6 mT low-frequency PEMFs promote ROB differentiation through the cAMP/PKA signaling pathway.

Key words: osteoporosis; pulsed electromagnetic fields; osteoblasts; cAMP/PKA signal pathway

Introduction

As the population structure continues to age, the prevention and treatment of osteoporosis has become a major global health concern. While vitamin D supplementation, increased calcium intake, and estrogen replacement therapy have demonstrated efficacy, these approaches carry increased risks of hypercalcemia and breast cancer. Electromagnetic fields, as a non-invasive, painless, and convenient physical stimulus, have attracted considerable attention. Studies have shown that electromagnetic fields can improve bone mineral density and biomechanical properties in experimental animals, reduce bone loss, and increase bone mineral deposition in osteoporotic patients. However, the underlying mechanisms remain incompletely understood and require further investigation.

The G protein-coupled cAMP/PKA signaling pathway participates in regulating numerous cellular functions. Upon binding of extracellular signaling molecules to their receptors, associated G proteins are activated, leading to adenylate cyclase activation and increased levels of the second messenger cAMP. cAMP then

binds to regulatory subunits of PKA, releasing catalytic subunits that directly or indirectly modulate various cellular activities. Research has demonstrated that the cAMP/PKA/CREB pathway is involved in osteogenic differentiation of human bone marrow mesenchymal stem cells, regulates MC3T3 osteoblastic differentiation, promotes bone sialoprotein and osteocalcin gene expression, and that specific knockout of the G protein -subunit in experimental animals significantly reduces trabecular bone number. This study investigated whether electromagnetic fields promote bone formation through the G protein-coupled cAMP/PKA signaling pathway to elucidate the molecular mechanisms underlying electromagnetic field-induced bone formation.

Materials and Methods

1.1 Low-Frequency Electromagnetic Field Cell Treatment Device

The low-frequency electromagnetic field cell treatment device used in this study was developed by our research group (Patent No.: ZL201110423102.0). The system comprises a computer control module, digital-to-analog conversion module, signal amplification module, coils, magnetic field sensor, and temperature sensor, capable of generating uniform electromagnetic fields including sine, triangle, square, pulse, and sawtooth waves with frequencies ranging from 5-200 Hz and intensities precisely adjustable from 0.0-9.0 mT. The device ensures that all cells within a 60 mm culture dish receive identical electromagnetic field exposure. During experiments, the coil was placed inside the cell culture incubator and connected to external components via cables [Figure 1: see original paper].

1.2 Materials

SPF-grade Wistar rats within 48 h of birth were obtained from the Animal Experiment Center of Gansu University of Chinese Medicine (Certificate No.: SCXK(Gan)2004-0006-152). -MEM medium and type I collagenase were from Gibco (USA); fetal bovine serum from Lanzhou Minhai Bio-Company; trypsin from Xi' an Kehao Biological Engineering; rat cAMP ELISA kit from R&D (USA); p-PKA antibody, PKA antibody, RUNX-2 antibody, OSX antibody from Abcam (USA); -Actin antibody from Bioworld (USA); DDA and KT5720 from Sigma (USA); dimethyl sulfoxide (DMSO) from AMRESCO (USA); alkaline phosphase (ALP) assay kit from Nanjing Jiancheng Bioengineering Institute; diethypyrocarbonate (DEPC), SDS-PAGE gel preparation kit, 5× protein loading buffer, BCA protein concentration assay kit, and ECL Plus hypersensitive luminescent solution from Beijing Solarbio Science & Technology; RNAiso Plus Reagent, Prime ScriptTM reagent Kit, and PCR amplification kit from Takara Bio.

1.3 Isolation and Culture of Rat Calvarial Osteoblasts

As described in reference [11], five Wistar rats within 48 h of birth were sterilized in 75% ethanol and euthanized. Calvaria were harvested, cut into approximately

2 mm² bone fragments, washed with PBS, and placed in culture flasks. The fragments were digested with 0.25% trypsin at 37°C for 10 min, after which the digestion solution was discarded. Subsequently, 0.1% type I collagenase was applied at 37°C for three 20-min digestion cycles. The collected digestion solutions were pooled in culture flasks containing medium, filtered through a 200-mesh cell strainer, and centrifuged at 1000 r/min for 5 min. The supernatant was discarded, and cell pellets were resuspended in culture medium, adjusted to a density of 3×10 cells/mL, and seeded into 90 mm culture dishes. Cells were cultured at 37°C in a 5% CO incubator and passaged upon reaching >90% confluence for experimental use.

1.4 Intracellular cAMP Concentration Measurement

Passage 1 ROBs were seeded at 3×10 /mL density in 60 mm culture dishes. Upon reaching near-confluence, cells were exposed to low-frequency electromagnetic fields for 0, 5, 10, 20, 40 min, 1 h, or 2 h. Culture medium was removed, cells were washed three times with ice-cold PBS, and lysed with 500 µL of 0.1 mol/L HCl at room temperature. Cell lysates were collected, homogenized, and centrifuged at 12,000 r/min for 10 min at 4°C. The supernatant was collected, and cAMP concentration was measured using an ELISA kit according to the manufacturer' s instructions.

1.5 Protein Expression Analysis

Passage 1 ROBs were seeded at 3×10 /mL density in 60 mm culture dishes. Upon reaching near-confluence, cells were treated under various conditions: exposure to 50 Hz 0.6 mT PEMFs for 0, 10, 20, 40 min, 1 h, or 2 h to detect p-PKA and PKA protein expression. Additional experimental groups included control, PEMF treatment alone, KT5720 alone, and KT5720+PEMF treatment groups. The control group received DMSO equivalent to the KT5720 volume without further treatment. The PEMF group was exposed to 50 Hz 0.6 mT PEMFs for 1.5 h daily. The KT5720 group was treated with 10 µmol KT5720. The KT5720+PEMF group received 10 µmol KT5720 concurrent with daily 1.5 h PEMF exposure. After 3 consecutive days, OSX, RUNX-2, and -actin protein expression was assessed. Culture medium was removed, cells were washed three times with ice-cold PBS, and lysed with 400 L PIRA protein lysis buffer. Lysates were centrifuged at 12,000 r/min for 30 min at 4°C, and supernatants were collected. Protein concentration was determined using 25 L of each sample, and 5× protein loading buffer (1/4 volume) was added to the remainder. Samples were boiled for 10 min, and 20 g of protein was subjected to SDS-PAGE gel electrophoresis followed by transfer to PVDF membranes. Membranes were blocked with 5% skim milk for 2 h at room temperature, incubated overnight at 4°C with primary antibodies (p-PKA 1:500, PKA 1:1000, OSX 1:800, RUNX-2 1:600, -Actin 1:1000), washed four times with TBST (8 min each), incubated with secondary antibody (1:10,000) for 2 h at room temperature with shaking, washed again four times with TBST (8 min each), and visualized using ECL

Plus hypersensitive luminescent solution in a darkroom. Images were captured and analyzed using IPP software.

1.6 ALP Activity Assay

Passage 1 ROBs were seeded at 3×10 /mL density in 30 mm culture dishes. Experimental groups included control, PEMF treatment alone, DDA alone, and DDA+PEMF treatment groups. Upon reaching near-confluence, cells received respective treatments: the control group received DMSO equivalent to the DDA volume without further treatment; the PEMF group was exposed to 50 Hz 0.6 mT PEMFs for 1.5 h daily; the DDA group was treated with 10 μmol DDA; and the DDA+PEMF group received 10 μmol DDA concurrent with daily 1.5 h PEMF exposure. ALP activity was measured after 3, 6, and 9 days of treatment according to the kit protocol: culture medium was removed, cells were washed three times with PBS, 250 μL each of substrate solution and buffer were added, gently mixed, incubated at 37°C for 15 min, followed by addition of 750 μL chromogenic solution with gentle mixing, and absorbance was measured at 520 nm to calculate ALP activity.

1.7 Osteogenic Gene Transcription Analysis

Passage 1 ROBs were seeded at 3×10 cells/mL density in 60 mm culture dishes. Experimental groups included control, PEMF treatment alone, DDA alone, DDA+PEMF, KT5720 alone, and KT5720+PEMF groups. Upon reaching complete confluence, cells received respective treatments: the control group received DMSO equivalent to DDA/KT5720 volume; the PEMF group was exposed to 50 Hz 0.6 mT PEMFs for 1.5 h daily; the DDA group received 10 μmol DDA; the DDA+PEMF group received 10 μmol DDA with concurrent PEMF exposure; the KT5720 group received 10 μmol KT5720; and the KT5720+PEMF group received 10 μmol KT5720 with concurrent PEMF exposure. After 3 consecutive days, culture medium was removed, cells were washed three times with ice-cold PBS, and lysed with 1 mL RNAiso Plus Reagent on ice. Cell lysates were collected, mixed with 200 L chloroform, vortexed, and centrifuged at 12,000 r/min for 15 min at 4°C. The aqueous phase was transferred, mixed with an equal volume of isopropanol, gently inverted, and centrifuged at 12,000 r/min for 20 min at 4°C. The supernatant was discarded, the pellet was washed with 1 mL freshly prepared 75% ethanol in DEPC-treated water, centrifuged at 12,000 r/min for 15 min at 4°C, and the pellet was dissolved in appropriate volume of DEPC water. RNA quality was assessed by measuring absorbance at 230, 260, 280, and 320 nm using a UV spectrophotometer. Reverse transcription was performed using Prime ScriptTM reagent Kit according to RNA concentration: 37°C for 15 min, 85°C for 5 s. The resulting cDNA was amplified using a PCR amplification kit: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Primer sequences are listed in . Data were analyzed using the 2^(-ΔΔCT) method.

TABLE:1 Primer sequences | Gene | GeneBank No. | Primer sequences | |—|

———-|————| | Gapdh | NM_017008.3 | Forward: 5'-GGCACAGTCAAGGCTGAGAATG-3' Reverse: 5' -ATGGTGGTGAAGACGCCAGTA-3' | | Bmp-2 | NM_017178 | Forward: 5'-ACCGTGCTCAGCTTCCATCAC-3'Reverse: 5'-TTCCTGCATTTGTTCCCGAAA-3'| | Collagen-1 | NM_001037632.1 | Forward: 5'-GCCTACTTACCCGTCTGACTTT-3'Reverse: 5'-GCCCACTATTGCCAACTGC-3'| | Osx | NM_053356 | Forward: 5'-TTCCCGGTGAATTCGGTCTC-3'Reverse: 5'-ACCTCGGATTCCAATAGGACCAG-3' |

1.8 Statistical Analysis

All data were analyzed using SPSS 20.0 statistical software. Inter-group differences were evaluated using one-way ANOVA, with multiple comparisons performed using the LSD method. Results are expressed as mean ± standard deviation. $P<0.05$ was considered statistically significant, and $P<0.01$ was considered highly statistically significant.

Results

2.1 Changes in Intracellular cAMP Concentration Following PEMF Treatment

As shown in [Figure 2: see original paper], intracellular cAMP concentration in ROBs remained unchanged after 5 and 10 min of continuous PEMF exposure, but increased significantly at 20 min compared to the control group ($P<0.01$). The elevation persisted until 40 min, then declined rapidly. By 1 h, cAMP levels showed no statistical difference from controls, but increased again at 2 h ($P<0.05$), demonstrating that low-frequency PEMFs elevate the second messenger cAMP concentration.

2.2 Changes in p-PKA Levels Following PEMF Treatment

To determine whether elevated cAMP concentration affects PKA phosphorylation, we examined p-PKA levels at various time points after PEMF exposure. As shown in [Figure 3: see original paper], p-PKA levels transiently decreased initially, then increased significantly at 20 and 40 min compared to controls (**$P<0.01$), returned to control levels, and increased again at 2 h (**$P<0.01$). This pattern mirrors the changes in cAMP concentration, indicating that PEMF-induced cAMP elevation triggers PKA phosphorylation.

2.3 Effect of DDA on ALP Activity Changes After PEMF Treatment

To clarify the relationship between PEMF-induced osteogenic differentiation and the second messenger cAMP, we measured ALP activity in ROBs after various treatments. As shown in [Figure 4: see original paper], ALP activity was significantly elevated in the PEMF treatment group at 3, 6, and 9 days compared to controls (**$P<0.01$, *$P<0.05$). DDA treatment alone did not affect ALP activity, but inhibition of AC activity with DDA markedly attenuated the

PEMF-induced increase in ALP activity, with statistically significant differences from the PEMF-only group (##P<0.01, #P<0.05). These results demonstrate that PEMF-promoted ALP activity elevation is closely associated with cAMP concentration changes.

2.4 Effect of DDA on Osteogenic Gene Transcription After PEMF Treatment

We examined osteogenic gene transcription levels in ROBs after various treatments. As shown in [Figure 5: see original paper], PEMF exposure significantly increased expression of Osx, Col-I, and Bmp-2 compared to controls (**P<0.01, *P<0.05). DDA treatment alone had no obvious effect on osteogenic gene transcription, but inhibition of AC activity with DDA markedly reduced PEMF-induced enhancement of Osx, Col-I, and Bmp-2 transcription, indicating that PEMF-mediated osteogenic gene transcription is associated with cAMP elevation.

2.5 Effect of KT5720 on Osteogenic Gene Transcription After PEMF Treatment

To investigate the relationship between PEMF osteogenic effects and PKA phosphorylation, we used the PKA-specific inhibitor KT5720 to pretreat ROBs. As shown in [Figure 6: see original paper], PEMF significantly increased expression of osteogenic genes Osx, Col-I, and Bmp-2 (**P<0.01, *P<0.05). KT5720 alone did not affect osteogenic gene transcription, but inhibition of PKA activity abolished PEMF-induced enhancement of osteogenic gene transcription (##P<0.01), demonstrating that the cAMP downstream effector PKA participates in PEMF-promoted osteogenic gene transcription.

2.6 Effect of KT5720 on Osteogenic Protein Expression After PEMF Treatment

Since KT5720 attenuated PEMF-induced changes in osteogenic gene transcription, we investigated whether it also affects protein expression using Western blotting. As shown in [Figure 7: see original paper], PEMF treatment increased osteogenic protein expression (**P<0.01). KT5720 alone did not affect protein expression, but it inhibited PEMF-induced enhancement of osteogenic protein expression (##P<0.01), confirming that PKA is involved in PEMF-induced osteogenic protein expression.

Discussion

Low-frequency PEMFs have been used clinically for fracture healing for nearly half a century, with numerous studies demonstrating their ability to promote bone formation. Our research group previously found that 50 Hz 0.6 mT PEMFs significantly promote ROB mineralization and maturation, though the precise mechanisms remain unclear. As a second messenger, cAMP is widely involved

in regulating cellular functions. Studies have shown that small molecule cAMP analogs can specifically activate PKA to promote MC3T3-E1 mineralization, and the cAMP/PKA pathway participates in icariin-induced bone formation. Through a series of experiments, we have demonstrated that the cAMP/PKA signaling pathway is involved in PEMF-induced bone formation.

Intracellular cAMP concentration is tightly controlled to precisely regulate cellular functions. Our analysis of cAMP and p-PKA levels after PEMF treatment revealed that cAMP concentration increased significantly at 20 min, remained elevated until 40 min, then decreased before rising again at 2 h. PKA phosphorylation showed a similar pattern, with significant increases at 20 and 40 min, a return to baseline, and another elevation at 2 h, confirming that PEMFs activate the cAMP/PKA signaling pathway. Inhibition of cAMP synthesis with DDA significantly reduced PEMF-induced increases in the early osteogenic differentiation marker ALP and decreased transcription of osteogenic genes Osx, Col-I, and Bmp-2, indicating that PEMFs regulate ROB osteogenic differentiation through the second messenger cAMP. Similarly, inhibition of PKA phosphorylation with KT5720 attenuated PEMF-induced enhancement of osteogenic gene transcription and protein expression, demonstrating that the cAMP downstream effector PKA also participates in PEMF-promoted bone formation.

As a ubiquitous and important signal transduction mechanism in organisms, the cAMP/PKA pathway regulates cellular functions through multiple mechanisms and plays a crucial role in bone metabolism. Our study reveals that 50 Hz 0.6 mT PEMFs promote bone formation by activating the cAMP/PKA pathway. However, other studies have shown that this pathway may play opposite roles in bone marrow mesenchymal stem cell differentiation, promoting adipogenic differentiation while inhibiting osteogenic differentiation and participating in osteoclast differentiation. Therefore, further research is needed to elucidate how PEMF-induced bone formation through the cAMP/PKA pathway is regulated within the complex and highly controlled metabolic network and how it interacts with other signaling pathways.

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Note: Figure translations are in progress. See original paper for figures.